

10/52744

PCT/DK 03/00590



REC'D 20 OCT 2003

WIPO PCT

Kongeriget Danmark

Patent application No.: PA 2002 01347

Date of filing: 12 September 2002

Applicants:
(Name and address) Nuevolution A/S
Rønnegade 8, 5.
DK-2100 København Ø
Denmark

Title: Proximity-aided synthesis of templated molecules.

IPC: -

This is to certify that the attached documents are exact copies of the above mentioned patent application as originally filed.

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)



Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

07 October 2003

Bo Z. Tidemann
Bo Z. Tidemann



PATENT- OG VAREMÆRKESTYRELSEN

Title

Proximity-aided synthesis of templated molecules

Technical Field of the Invention

5 The present invention relates to a method synthesising a bifunctional complex. The bifunctional complex comprises a template conjugated to a templated molecule. The templated molecule is synthesised using the directions programmed in the template. The invention also relates to a library comprising different bifunctional molecules. The library of different complexes
10 can be used in the quest for new drug candidates. In certain aspects of the present invention it is possible to use the library of complexes for molecular evolution.

Background

15 The generation of molecules carrying new properties remains a challenging task. Recently, a number of procedures have been suggested that should allow a more efficient generation and screening of a larger number of molecules. The approaches taken involve the encoding and/or templating of molecules other than natural biopolymers such as peptide, RNA and DNA.
20 These approaches allow the researcher to generate and screen a huge number of molecules in a short time. This should lead to better molecules carrying the desired properties.

The central dogma of biology describes the one-way flow of information from
25 DNA to RNA to protein. Recently, methods such as phage display, peptides-on-plasmids, ribosome display, and mRNA-protein fusion have been developed, allowing the transfer of information from the level of protein/peptide to RNA or DNA. This has enabled the use of molecular evolution to be applied on huge numbers of peptides that are exposed to an enrichment process,
30 where after the enriched pool of molecules (enriched for a particular feature, such as binding to receptor protein) are amplified, by exploiting information flow from the peptide to DNA and then amplifying the DNA.

More recently, approaches have been developed that allow the encoding of polypeptides and other biochemical polymers. An example of this approach is disclosed in US 5,723,598, which pertains to the identification of a biochemical polymer that participates in a preselected binding interaction with a target to form a binding reaction complex. The prior art method encompasses the generation of a library of bifunctional molecules. One part of the bifunctional complex is the biochemical polymer and the other part is an identifier oligonucleotide comprising a sequence of nucleotides which encodes and identifies the biochemical polymer. Following the generation of the library of the bifunctional molecules, a partitioning with respect to affinity towards the target is conducted and the identifier oligonucleotide part of the bifunctional molecule is amplified by means of PCR. Eventually, the PCR amplicons are sequenced and decoded for identification of the biochemical polymer. This approach does not, however, allow one-pot amplification of the library members. Furthermore, the sequence of nucleotides serves to identify the biochemical molecule only after a laborious sequencing process. Thus the flow of information from the identifier sequence to the biochemical polymer is restrained.

Halpin and Harbury have in WO 00/23458 suggested an improvement to the approach stipulated immediately above, wherein the molecules formed are not only identified but also directed by the nucleic acid tag. The approach is based on the traditional split-and-combine strategy for synthesis of combinatorial libraries comprising two or more synthetic steps. A plurality nucleic acid templates are used, each having at one end a chemical reactive site and dispersed throughout the stand a plurality of codon regions, each of said codon regions in turn specifying different codons. Separately, each of the strands, identified by a first codon region, is reacted at the chemical reaction sites with specific selected reagents. Subsequently, all the strands are pooled and subjected to a second partitioning based on a second codon region. The split-and-combine method is conducted an appropriate number of times to pro-

duce a library of typically between 10^3 and 10^6 different compounds. The split-and-combine method is cumbersome and generates only a relatively small library.

- 5 A more direct method for templating non-natural small molecule libraries has been suggested by Gartner and Liu (J. Am. Chem. Soc. 2001, 123, 6961-6963). While not supported by experimental evidence or otherwise enabled, a method is illustrated in a drawing which starts with a template nucleic acid having attached to an end thereof a molecular moiety carrying a reactive
- 10 groups. The template is divided into a number of coding regions, wherein each of the codon regions specifies a specific codon. A set of building blocks consisting of a bifunctional molecule having an anti-codon part and a reagent part are annealed to the plurality of codons of the first codon region. Subsequently, the reagent part reacts with the one of the reactive groups of the mo-
- 15 lecular moiety attached to one end of the template and the reagent part and the anti-codon part of the building block is separated. A second reaction step is initiated by the annealing of a second set of building blocks capable of hybridising to the second codon region and is followed by reaction and cleavage of the bifunctional building block. The method may be repeated a suitable
- 20 number of times to produce a library of scaffolded entities covalently connected to one end of the template which templated the synthesis thereof. The process may be performed as a one-pot approach without the need for a splitting step, however suffer from the drawback that the distance from the reactive group of a building block to the reactive group of the scaffold varies
- 25 depending on the position of the codon region of the template. This drawback becomes even more relevant, when considering that the local concentration of one of the reactive groups (e.g., the reactive group carried by the building block) relative to the other reactive group (i.e., the reactive group of the scaffold) decreases 1000-fold, when the distance between the reactive groups is
- 30 increased 10-fold. The present invention provides an attempt to eliminate or reduce this drawback.

Summary of the Invention

The present invention concerns a method for synthesising a bifunctional complex comprising a template-directed molecule and a template for the synthesis thereof, the method comprising the steps of:

- 5 a) providing a template comprising two or more codons in sequence, a first part of a molecular affinity pair, and a reactive group,
- b) providing two or more building blocks, each building block comprises
 - 10 i) an anti-codon capable of recognising a codon of the template,
 - ii) a functional entity comprising at least one reactive group,
 - iii) a linker connecting the anti-codon and the functional entity,
 wherein building blocks having anti-codons intended to interact with codons of the template distal to the reactive group comprise as a section of the linker a second part of the molecular affinity pair,
- 15 c) contacting the template with a building block under conditions which allow specific hybridisation of the anti-codon of the building block to the codon of the template, and under conditions ensuring assembling of the parts of the molecule pair, if present,
- 20 d) obtaining a connection between the functional entity of the building block and the template by a reaction involving the template reactive group and the functional entity reactive group,
- e) cleaving a linkage to obtaining a nascent templated molecule,
- f) separating the parts of the molecular affinity pair,
- 25 g) repeating, for a building block having an anti-codon capable of hybridising to a new codon, steps c) to f) one or more times.

The molecular affinity pair is composed of two parts which has affinity for each other under certain environmental conditions. The essential property of
 30 the molecular affinity pair is that the two parts are capable of interacting in order to assemble the molecular affinity pair. In the biotechnological field a variety of interacting molecular parts are known which can be used as the

molecular affinity pair according to the invention. Examples include, but are not restricted to protein-protein interactions, protein-polysaccharide interactions, RNA-protein interactions, DNA-DNA interactions, DNA-RNA interactions, RNA-RNA interactions, biotin-streptavidin interactions, enzyme-ligand interactions, antibody-ligand interaction, protein-ligand interaction, ect.

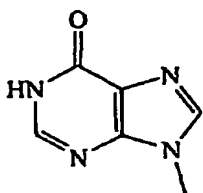
The interaction between the molecular affinity parts may result in a strong or a weak bonding. If a covalent bond is formed between the parties of the affinity pair the binding between the parts can be regarded as strong, whereas the establishment of hydrogen bondings, interactions between hydrophobic domains, and metal chelation in general results in a weak bonding. In general relatively weak bonding is preferred. In a preferred aspect of the invention, the first part of the affinity pair is capable of reversibly interacting with the second part of the affinity pair so as to provide for an attachment or detachment of the parts in accordance with the changing conditions of the media.

In a preferred aspect of the invention, the molecular affinity pair is based on an interaction between nucleotides, i.e. the first part of the affinity pair is a sequence of nucleotides and the second part of the affinity pair is a sequence of nucleotides capable of hybridising to the first part of the affinity pair. The first part of the affinity pair is a part of the template and may comprise an oligonucleotide having nucleobases selected among the natural occurring nucleobases, i.e. adenine, cytosine, guanine, thymine, and uracil which are attached to a backbone, such as a repetitive sequence of (deoxy)ribose-phosphate units. The second part of the affinity pair can be an oligonucleotide having nucleobases which complements and is specifically recognised by the first part, i.e. in the event the first part contains cytosine, the second part contains guanine and visa versa, and in the event the first part contains thymine or uracil the second part contains adenine. In one aspect of the invention it is preferred however, that at least some of the nucleobases of the second part of the affinity pair are non-specific base-pairing nucleobases. Non-specific base-pairing nucleobases are bases which, when attached to a

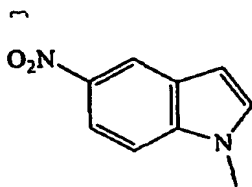
backbone, are able to pair with at least two of the five naturally occurring nucleobases mentioned above. Preferably, the base pairing between the two or more natural nucleobases and the non-specifically base-pairing nucleobase occurs essentially iso-energetically, i.e. the bonds formed have a strength of the same order. The term "non-specifically base-pairing nucleobase" is used herein interchangeably with the term "universal base".

In natural tRNA the nucleobase inosine is found. Inosine has the ability to hybridise non-specifically with three of the nucleobases, i.e. cytosine, thymine, and adenine. Other synthetic compounds having the same ability of non-specifically base-pairing with natural nucleobases have been formed and includes among others the compounds depicted below

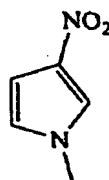
Examples of Universal Bases:



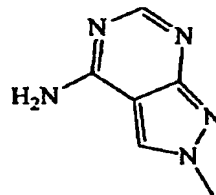
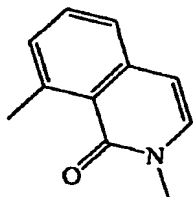
Inosine



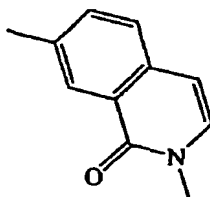
5-Nitroindole



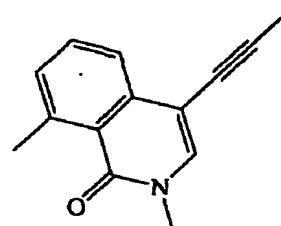
3-Nitropyrrole

N⁸-8aza-7deazaadenine

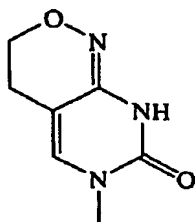
MICS



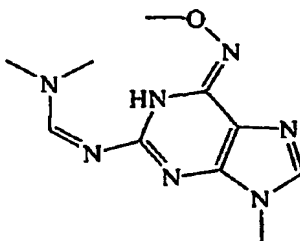
5MICS



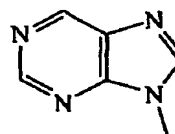
PIM



dP



dK



Nebularine

The use of universal bases in the present method has an advantage in the generation of a library because the codons serving as coding entities may also serve as the first part of the affinity pair. In an aspect of the invention,

therefore, the first part of the molecular affinity pair is comprised by the two or more of the codons in sequence. Preferably, the nucleotide sequence of the first part of the molecular affinity pair comprise at least a part of the nucleotide sequence of the codon proximal to the reactive group of the template. A building block oligonucleotide hybridised to a template oligonucleotide may involve more than one molecular affinity pair. A further molecular affinity pair may be placed between the functional entity and the anti-codon of a building block or in a tail section behind the anti-codon. The further molecular affinity pair may provide for a stronger attachment of the building block to the template. The proximal arrangement of the molecular affinity pair compulsory present ensures that the local concentration of reactive groups which are subjected to reaction is increased considerably, thus increasing the probability that a connection will be formed.

The codons of the template may be any biochemical entity with an ability to be recognized specifically by another entity. It is preferred, however, that the codon is a sequence of nucleotides. The sequence of nucleotides carries a series of nucleobases on a back bone. The nucleobases of the codons may be any chemical entity able to be specifically recognized by a complementing entity. The nucleobases are usually selected from the natural nucleobases (adenine, guanine, uracil, thymine, and cytosine) but also the other nucleobases obeying the Watson-Crick hydrogen-bonding rules may be used, such as the synthetic nucleobases disclosed in US 6,037,120.

The codon may be a single nucleotide. In the generation of a library, this will allow for the incorporation of four different functional entities into the template-directed molecule. However, to obtain a higher diversity a codon preferably comprises at least two and more preferred at least three nucleotides. Theoretically, this will provide for 4^2 and 4^3 , respectively, different functional entities. The codons will usually not comprise more than 100 nucleotides. It is preferred to have codons with a sequence of 3 to 30 nucleotides.

The at least two codons of the template are arranged in sequence, i.e. next to each other and may be separated by a spacer group. Depending on the template-directed molecule intended to be formed, the template may comprise further codons. Each of the further codons may be separated by a suitable spacer group. Preferably, all or at least a majority of the codons of the template are arranged in sequence and each of the codons are separated from a neighbouring codon by a spacer group. Generally, it is preferred to have more than two codons on the template to allow for the synthesis of more complex template-directed molecules. In a preferred aspect of the invention the number of codons of the template is 2 to 100. Still more preferred is templates comprising 3 to 10 codons.

The spacer sequence may serve various purposes. In one setup of the invention, the spacer group identifies the position of a codon. Usually, the spacer group either upstream or downstream of a codon comprises information which allows determination of the position of the codon. The spacer group may also or in addition provide for a region of high affinity. The high affinity region will ensure that the hybridisation of the template with the anti-codon will occur in frame. Moreover, the spacer sequence adjusts the annealing temperature to a desired level.

A spacer sequence with high affinity can be provided by incorporation of one or more nucleobases forming three hydrogen bonds to a cognate nucleobase. An example of a nucleobase having this property is guanine. Alternatively, or in addition, the spacer sequence may be subjected to back bone modification. Several back bone modifications provides for higher affinity, such as 2'-O-methyl substitution of the ribose moiety, peptide nucleic acids (PNA), and 2'-4' O-methylene cyclisation of the ribose moiety, also referred to as LNA (Locked Nucleic Acid).

The template may comprise flanking regions. One of the flanking regions can in an aspect of the invention serve to immobilize the template to a surface of a solid support such as a microarray. In another aspect of the invention the

flanking region can encompass a signal group, such as a fluorophore or a radio active group, to allow a direct detection of the presence of the template. The flanking regions can also serve as priming sites for an amplification reaction, such as PCR.

- 5 The template may also be immobilised on a solid support, such as a bead or matrix material by incorporating a biotin group in the template and subsequent coupling to a streptavidin coated solid support. Various other immobilisation methods are known to the skilled person, including coupling of the template to an antibody and immobilising the conjugate to a solid support
- 10 coated with the appropriate antigen. In a preferred aspect, the priming site of the template serves the dual purpose of participating in an amplification reaction and as the means for immobilisation. The immobilisation can be effected, e.g. by treatment of the template comprising the priming site with a solid support comprising oligonucleotide sequences complementary to the priming
- 15 site.

- The reactive group of the template may be provided by various means and in a variety of ways. In one aspect, the reactive group of the template is covalently attached to the template. The covalent attachment of the reactive group usually entails that the template-directed molecule is formed at or in the vicinity of said reactive group. The final template-directed molecule is
- 20 thus covalently attached to the template which directed and encoded the synthesis thereof. In the event a library is formed which comprises a plurality of complexes prepared in accordance with the invention, high stringency conditions for a selection procedure may be used without the risk of separating the
- 25 template-directed molecule from the template.

- In another aspect of the invention, the reactive group of the template is non-covalently attached to the template. Usually, the non-covalent attachment involves hydrogen bonds and hydrophobic interaction. Notably, the non-covalent attachment involves a hybridisation reaction between oligonucleotides or a part thereof. In a preferred embodiment, the reactive group of the
- 30 template is attached to a sequence of nucleotides, which complements a se-

quence of nucleotides of the template. The complementing sequence having attached the reactive group can serve as an anchor, i.e. to tie the nascent template-directed molecule to the template. Usually, the complementing sequence of the anchor has an annealing temperature higher than each of the building blocks to ensure attachment of the anchor even under condition which detaches the building blocks.

The reactive group may be linked to the template through a selectively cleavable linker, which enables the separation of the template-directed molecule from the template at a time decided by the experimenter. The reactive group can also be a part of a functional entity or nascent template-directed molecule which, possibly in amended form, appears in the final templated molecule. The reactive group can also be a part of a scaffold, i.e. a molecular entity comprising more than one reactive group. Furthermore, the reactive group of the template may be in a pro-form that has to be activated before the method of the invention is initiated.

In the aspect of the invention relating to the generation of a library, it may be desired to couple the reactive group of the template to an anti-codon complementing a (further) codon on the template, thus making it possible to have more than a single kind of reactive groups present in the media. Alternatively, a functional entity or scaffold comprising the reactive group(s) may be varied. In an aspect of the invention, the template comprises two regions of codons, the two or more codons in sequence of step a) belonging to the first region and the second region of codons comprising one or more codon(s) for initial attachment of the reactive group of the template. During the propagation part of the method it is possible to design the synthesis such, that each new building block incorporated is alternating annealed to the two codon regions. Preferably the two codon regions comprise the same number of codons. The alternating incorporation of building blocks on the template has the further advantage that the nucleotide sequences used as anti-codon and linker may be shorter for the generation of a template-directed molecule having participated in an appropriate number of reactions.

It is within the capability of the skilled person in the art to construct the desired design of an oligonucleotide. When a specific annealing temperature is desired it is a standard procedure to suggest appropriate compositions of nucleic acid monomers and the length thereof. The construction of an appropriate design may be assisted by software, such as Vector NTI Suite or the public database at the internet address

<http://www.nwfsc.noaa.gov/protocols/oligoTMcalc.html>.

The conditions which allow specific hybridisation of the codons and the anticodons are influenced by a number of factors including temperature, salt concentration, type of buffer, and acidity. It is within the capabilities of the person skilled in the art to select appropriate conditions to ensure that the contacting between the templates and the building blocks are performed at hybridisation conditions. The temperature at which two single stranded oligonucleotides forms a duplex is referred to as the annealing temperature or the melting temperature. The melting curve is usually not sharp indicating that the annealing occurs over a temperature range. The second derivative of the melting curve is used herein to indicate the melting temperature.

When the template is linear, the first part of the molecular affinity pair is usually arranged between the active codon and the reactive group of the template to provide for a closer proximity between the reactive groups. More preferred, the first part of the molecular affinity pair is arranged proximal relative to the template reactive group. To increase the proximity, the distance between the nucleotide carrying the template reactive group and the nucleotide carrying the reactive group of the building block is no more than two nucleotides. More preferred the distance is no more than a single nucleotide, and still more preferred there is no distance, i.e. the nucleotide carrying the reactive group are abutted or annealed to each other.

The second part of the molecular affinity pair is positioned in the building block. The second part of the molecular affinity pair may be dispensed with in the event the codon to which the building block is attached to is close to the

- template reactive group, or expressed in another way, the anti-codon of the building block may be identical to the second part of the molecular affinity pair. Building blocks having anti-codons intended to interact with codons distal to the template reactive group comprise as a section of the linker the second part of the molecular affinity pair. The term "distal" is to be understood as the case in which the active codon, i.e. the codon hybridised to the anti-codon of the building block, is interspaced relative to the template reactive group with one or more inactive codon(s).
- Usually, the system is designed to provide for a higher affinity between the codon and the anti-codon compared to the affinity of the molecular affinity pair because it is of importance that the specific recognition between codon and anti-codon is effective.
- The second part of the molecular affinity pair in the linker of the building block is preferably arranged proximal to the functional entity to increase the proximity between the building block reactive group and the template reactive group. More preferred the second part of the molecular affinity pair is spaced from the nucleotide carrying the functional entity by 0 to two nucleotides.
- The functional entity of the building block serves the function of being a precursor for the structural entity eventually incorporated into the templated molecule. Therefore, when it in the present application with claims it is stated that a functional entity is transferred to a nascent template-directed molecule it is to be understood that not necessarily all the atoms of the original functional entity is to be found in the eventually formed template-directed molecule. Also, as a consequence of the reactions involved in the connection, the structure of the of the functional entity can be changed when it appears on the nascent templated molecule. Especially, the cleavage resulting in the release of the functional entity may generate a reactive group which in a subsequent step can participate in the formation of a connection between a nascent templated molecule and a functional entity.

The functional entity of the building block comprises at least one reactive group capable of participating in a reaction which results in a connection between the functional entity of the building block and the part of the template or complementing element hybridised to the template carrying the template reactive group. The connection is aided by one or more reactive groups of the functional entity. The number of reactive groups which appear on the functional entity is suitably one to ten. A building block featuring only one reactive group is used i.a. in the end positions of polymers, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffolds capable of being reacted further. Two or more reactive groups intended for the formation of connections, are typically present on scaffolds. A scaffold is a core structure, which forms the basis for the creation of multiple variants. The variant forms of the scaffold is typically formed through reaction of reactive groups of the scaffold with reactive groups of other building blocks, optionally mediated by fill-in groups or catalysts, under the creation of a connection between the entities. The functional entities to be connected to the scaffold may contain one, two or several reactive groups able to form connections.

The reactive group of the building block may be capable of forming a direct connection to a reactive group of the template or the reactive group of the building block may be capable of forming a connection to a reactive group of the template through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection.

The subsequent cleavage according to step e) can be performed in any appropriate way. In an aspect of the invention the cleavage involves usage of a reagent or and enzyme. The cleavage results in a transfer of the functional entity to the nascent template-directed molecule or in a transfer of the nas-

cent template-directed molecule to the functional entity of the building block. In some cases it may be advantageous to introduce new chemical groups as a consequence of linker cleavage. The new chemical groups may be used for further reaction in a subsequent cycle, either directly or after having been activated. In other cases it is desirable that no trace of the linker remains after the cleavage.

In another aspect, the connection and the cleavage is conducted as a simultaneous reaction, i.e. either the functional entity of the building block or the nascent template-directed molecule is a leaving group of the reaction. In general, it is preferred to design the system such that the connection and the cleavage occur simultaneously because this will reduce the number of steps and the complexity. The simultaneous connection and cleavage can also be designed such that either no trace of the linker remains or such that a new chemical group for further reaction is introduced, as described above.

It is important for the method according to the invention that at least one linker remains intact after the cleavage step. The at least one linker will link the nascent template-directed molecule to the template that directed the synthesis thereof. In case the method essentially involves the transfer of functional entities to a scaffold or an evolving polymer, the eventually scaffolded molecule or the polymer may be attached with a selectively cleavable linker. The selectively cleavable linker is designed such that it is not cleaved under conditions which result in a transfer of the functional entity to the nascent template-directed molecule.

The building blocks used in the method according to the present invention may be designed in accordance with the particular entities involved in the building block. As an example, the anti-codon may be attached to the second part of the molecular affinity pair with a polyethylene glycol (PEG) linker and the functional entity may be directly attached to the second part of the molecular affinity pair. In another and preferred example, the anti-codon, the

linker and the second part of the molecular affinity pair is a contiguous linear oligonucleotide. In the event, the anti-codon:codon hybrid and the molecular affinity pair is interspaced with one or more codons on the template, the linker is preferably provided with nucleotides having affinity towards the template nucleotides. In a preferred aspect of the invention, universal nucleobases are incorporated into the linker at the position of the interspacing inactive codons. If the anti-codon is arranged next to the functional entity, the linker may be a single linkage.

10 The attachment of the functional entity to the linker is preferably at a terminal nucleotide or a nucleotide 1 or two nucleotides down the oligonucleotide. The attachment of the functional entity can be at any entity available for attachment, i.e. the functional entity can be attached to a nucleotide of the oligonucleotide at the nucleobase, or the back bone. In general, it is preferred to attach the functional entity at the phosphor of the internucleoside linkage or at
15 the nucleobase.

In a preferred aspect of the invention, the reactive group of the functional entity is attached to the linker oligonucleotide. The reactive group is preferably
20 of a type which is able to create a connection to the template or the nascent template-directed molecule by either direct reaction between the respective reactive groups or by using a suitable fill-in group. The reactive group coupling the functional entity with the linker is preferably cleaved simultaneously with the establishment of the connection. The functional entity may in some
25 cases contain a second reactive group able to be involved in the formation of a connection in a subsequent cycle. The second reactive group may be of a type which needs activation before it is capable of participating in the formation of a connection.

30 The oligonucleotide linker may be distanced from the reactive group of the functional entity by a spacer moiety. The spacer may be designed such that the conformational spaced sampled by the reactive group is optimized for a

reaction with the template reactive group or a reactive group of a nascent template-directed molecule.

5 The design of building blocks comprising the anti-codon may be aimed at obtaining annealing temperatures in a specific range for all or some of the building block:template hybrids to ensure that the anti-codons have been annealed to the template before the functional entities are connected to each other through a chemical reaction. When the building blocks anneals to the template with essentially the same affinity it is necessary to add the building
10 blocks in each cycle, i.e. the contacting of the building blocks with the template involves separate addition of the individual building blocks.

15 In an aspect of the invention, the building blocks are designed such that the building block to be added to the template in the first cycle has an annealing temperature lower than the subsequent building blocks. By using a temperature for the connection step in a second or subsequent step which is higher the previous step it is possible to have only the intended building blocks annealed to the template, while the majority of previous spent or non-reacted building blocks will be single stranded. Optionally, a recovery step may be
20 used between each cycle to enrich the number of single stranded template available for annealing to a subsequent building block. The recovery step may involve the incorporation of biotin in the building block oligonucleotide and separation of the building blocks from the template using streptavidin coated beads at a temperature above the annealing temperature, as described elsewhere herein.
25

In another aspect of the invention, the annealing temperature of the individual building block:template hybrids is designed to be different. According to this aspect, all the building blocks may be added to the media simultaneously.
30 The separate steps of the method may be accomplished in the correct order by initially raising the temperature above the annealing temperature for all the hybrids and slowly decreasing the temperature until the first anti-codon an-

5 neal to its template. Following the connection of the functional entity to another functional entity or a nascent templated molecule, and the separation of the parts of the molecular affinity pair, the temperature is decreased sufficient for allowing another building block to anneal to the cognate codon. According to this aspect of the invention the method comprises addition of all, or a substantial amount of, the building blocks to the template and directing the contacting by step-wise decreasing the temperature.

10 After the cleavage step the parts of the molecular affinity pair are separated to allow for a subsequent building block to be attached by the molecular affinity pair to the template. Optionally, the cleavage step may be performed after the separation of the molecular affinity pair. In case the molecular affinity pair is a double stranded oligonucleotide, the parts of the affinity pair may be separated by increasing stringency, e.g. by increasing the temperature. In the alternative, the second part of the affinity pair carried by the building block, 15 can be degraded enzymatically or chemically as disclosed below.

20 After the reaction of a building block, e.g. by transfer of a functional entity to a scaffold, the anti-codon may remain annealed to the template during a subsequent cycle. However, it is in general preferred to remove the anti-codon of a reacted building block not harbouring the nascent template-directed molecule from the template prior to repetition of steps c) to f). The absence of the annealed anti-codon makes it possible to incorporate universal bases in the linker to obtain an affinity between the linker and the inactive previous used 25 codons.

30 The anti-codon can be removed using various techniques, such as separation from the template by increasing the stringency, typically by raising the temperature; partly or fully enzymatical digestion; or chemical degradation. The approach using increasing the stringency is the most simple to apply. However, in the event reannealing can occur or selective removal of the anti-

codon is desired, it can be contemplated to use enzymatic or chemical approaches or a mixture thereof.

5 A method for removal of spent building blocks, non-reacted building blocks and excess building blocks involves the incorporation of a partner of an affinity pair and withdrawal of said building block using the second partner. A suitable example is the incorporation of biotin in the building block oligonucleotide and capture of the biotin with streptavidin coated beads. More specifically, biotin is incorporated in the building block during the synthesis
10 thereof. Following the transfer step or alternatively the cleavage step of the invention, the mixture is treated with beads coated with streptavidin under conditions which allow for the coupling of streptavidin to biotin. Subsequently, the temperature is increased above the annealing temperature of the building block:template hybrid and the mixture is subjected to increased gravity, e.g
15 by spinning in a centrifuge. The supernatant will then comprise the template liberated from the building blocks. An alternative to the biotin-streptavidin coupling is the formation of a S-S bridge. As an example, the oligonucleotide comprising the anti-codon is provided with a -SH group, such as a reduced product of the C6 S-S thiol modifier (Glen Research# 10-1936-90). The -SH
20 group of the building block can be coupled to another -SH group on a solid support under oxidising conditions and the building block can be removed together with the solid support by spinning if the solid material is a bead or by elution if the solid support is a solid phase matrix of a column.

25 For some applications it may be of advantage to selectively degrade the anti-codon-containing oligonucleotide. Several methods are available for degradation of the RNA part of a DNA:RNA duplex. Accordingly, the template can be provided as a single stranded oligonucleotide and the anti-codon can be a single cognate RNA strand. The DNA:RNA duplex can then be degraded with
30 an enzyme selected from RNaseH, RNaseA, RNase 1. In the alternative, the RNA part of the RNA:DNA duplex can be degraded chemically by treatment under weak alkaline conditions (pH 9-10), or with aqueous $Pb(Ac)_2$.

If the internucleoside linker comprises a thiophosphate, the linker may be cleaved with iodine. Therefore, according to this approach, an oligonucleotide template, such as a DNA or RNA template having hybridised thereto an DNA or RNA anti-codon comprising a thiophosphate in the internucleoside linker can be treated with aqueous iodine or iodoethanol to cleave the anti-codon.

According to another approach, a strand may be cleaved in a duplex if a DNA monomer contains a uracil nucleobase by first treating the duplex with uracil-glycosylase to remove the uracil moiety and subsequently treating with weak acid. Yet another approach involves the inclusion of methyl phosphonate in the internucleoside linker and cleavage of the linker using piperidine, e.g. by treatment at 37°C for an hour with a piperidine concentration of 100mM.

The various methods of removal of the anti-codon from the template can be used in the selective degradation of anti-codons. The advantage of selective degradation is especially apparent when the nascent template-directed molecule as well as the building block is encoded for by the template. In one aspect, a scaffold is coded for by the template and building blocks are sequentially incorporated. By using any of the above methods it is possible selectively to remove the building block, including the anti-codon and the linker, while the anti-codon used for recognising the codon which codes for the scaffold remains attached to the template.

When a strategy is followed wherein the eventually produced templated molecule is attached to a template via a complementing element, which may and may not involve an anti-codon, the affinity is relatively weak because only hydrogen bondings and hydrophobic interactions tight the parts together. Therefore, in an aspect of the invention, the complementing element finally harbouring the templated molecule, may be attached to the template through a complementing element:template hybrid having a higher annealing temperature than the other codon:anti-codon hybrids of the template. Alterna-

tively, and in some applications preferably, the templated molecule is connected with the template which directed the syntheses thereof via a covalent link. The covalent link may be in addition to the hydrogen bondings or the covalent link may be a substitution. The presence of a covalent link allows for a more harsh chemical treatment of the complex. In one aspect of the invention, the covalent link is selectively cleavable to provide for a separation of the templated molecule from the complementary template.

The method according to the invention may, as a further step, involve the transfer of the templated molecule to an anchorage point on the template, or a sequence complementing the template, to establish an effective chemical connection between the template and the templated molecule. An effective coupling of the templated molecule to the template or a sequence complementary to the template can be desirable to allow for denaturing enrichment conditions or denaturing post-templating modification of the manufactured molecule. The anchorage may involve the presence of a reactive group on the templated molecule and a reaction partner on the template, whereby the reaction between these reactive groups will establish a covalent link. Alternatively, the anchorage point may be present on a complementary sequence hybridised to the template. In a preferred embodiment the complementing sequence has a higher annealing temperature than one or more of the building blocks, notably the terminal building block, to enable usage of a higher stringency during enrichment and, optionally, clearance of used building blocks.

The present invention also relates to a library of bifunctional complexes. The library is composed of a plurality of different complexes, such as at least 10^3 , 10^6 , 10^9 , 10^{12} , or 10^{15} different complexes. The plurality of different complexes is produced by initially providing a plurality of different templates as well as a plurality of building blocks. Each of the anti-codons of the building blocks is adapted so as to be capable of interacting with at least one codon of at least one template. The plurality of different templates is simultaneously

subjected to the method described herein above. The propagation part of the method may be repeated a desired number of times to evolve the templated molecule. Each repetition of the propagation is initiated by contacting the templates with a new subset of further building blocks.

5

The various different templates of the present invention are conveniently constructed to follow a general scheme. According to the scheme, a number of coding sections are provided on the template. In turn, each of the coding sections specifies one or more unique codons. Thus, a specific template
10 comprises a given number of unique codons. The plurality of templates can, taken as a whole, be characterized as a library comprising the total amount of the different combinations of unique codons possible, or any subset thereof. The coding sections are suitably positioned in a linear sequence, such that the individual coding sections are positioned immediately next to
15 each other, optionally, interspaced by a spacer sequence. In some embodiments, it may be of advantage to use a branched template to ensure proximity of reactive groups, the introduction of catalysts in the vicinity of the reactive groups or the introduction of as third reactant.

20

The unique codons of the templates are preferably composed of a sequence of nucleic acid monomers, such as nucleotides. Each codon is preferably unique in the sense that within the same coding section no other codons have an identical sequence and length of nucleic acid monomers. Preferably, a unique codon does not have a corresponding sequence anywhere in the
25 plurality of templates. To avoid hybridisation between individual templates it is also desirable to design each of the unique codons such that the complementary sequence thereof does not exist on any other templates.

30

The number of coding sections may be selected in accordance with inter alia the number of the desired final templated compounds, the building blocks available and the envisaged structure of the templated compound. According to the invention the number of coding regions is preferably at least 3 to

achieve the desired diversity. The upper limit for the number of coding regions has not yet been elucidated; however it is believed that a number exceeding 100 may give practical problems. Generally, it is preferred to use templates having between 2 and 50 coding regions, more preferably between 3 and 30 and still more preferred between 4 and 15.

Within each of the coding regions the number of unique codons may be selected according to the need for diversity. The number of unique codons in each of the coding regions may be similar or different. The number of unique codons can be as low as one. This may be the choice when a specific molecular entity is wanted in the evolving templated molecule. The upper limit for the number of unique codons may be chosen quite high as long as specific hybridisation of oligonucleotides of the anti-codons to their complements on the templates occurs. An example of an upper limit may be 10,000, but may be chosen below this limit or above according to the need.

As an example of a relatively small library, around 10^6 different complexes can be obtained for templates having 4 coding regions, wherein each coding region specifies 30 unique codons. If each of the unique codons must only be present once on the template, at least 120 different building blocks have to be provided. The plurality of templates and the building blocks may be used for the generation of a 4-mer compound, such as an alpha or beta peptide. A larger library of 10^{10} complexes may be prepared starting from templates having 5 coding regions and 100 unique codons within each coding region.

The library may be used for a variety of applications, including the search for compounds for use in therapeutic or diagnostic methods and plant protection compounds, like pesticides, fungicides etc. The library may comprise any number of complexes according to the invention.

One method to identify the most active compounds which can be used in e.g. therapeutic applications is to subject the library to an enrichment treatment.

According to one aspect of the invention an enrichment of a library of complexes comprising templated molecules with respect to a predetermined activity, comprises the steps of:

- 5 i) establishing a first library of complexes comprising templated molecules, said library being obtainable according to any of the methods of the invention,
- ii) exposing the library to conditions enriching the library with complexes having the predetermined activity,
- 10 iii) amplifying the complexes of the enriched library,
- iv) optionally, repeating step ii) to iii), and
- v) obtaining an enriched library having a higher ratio of complexes comprising templated molecules with the predetermined activity.

The amplification step is normally preferred, though not always necessary. Especially, when several cycles of enrichments are conducted it is of advantage to make an amplification to obtain sufficient complexes. In a preferred aspect of the invention, the amplification of the complexes of the enriched library comprises the steps of contacting the library of complexes with amplification means, amplifying the templates or the complementing templates, and conducting the method according to the invention using the amplification product as templates. The amplification means can be any of the nucleic acid amplification means suitable for the amplification of the template, such as PCR. Preferably, the amplification of the complex comprises a 10^1 to 10^{15} -fold amplification.

25 To allow for multiple enrichment cycles the steps ii) and iii) are repeated at least 2, 3, 5 times, such as at least 10 times, such as at least 15 times. The complexes may be identified after the completion of each cycle or may be only be identified after the last cycle. There is no explicit need for
30 intermediate identifications as the amplification can be performed without knowing the sequence of the template or a sequence complementing the template, if the template or the complement thereof is provided with suitable

primer regions. The identification after the enrichment process involves the determination of the sequence of the template and/or the structural determination of the templated molecule and/or the entire complex having the predetermined activity.

5

Preferably, the conditions enriching the library comprise contacting a binding partner to the templated molecules of interest. The binding partner may be in solution or may be directly or indirectly immobilised on a support. The enrichment is in general performed using an affinity or activity assay. In one aspect of the invention, the enrichment is conducted by screening for complexes having an affinity for – or an effect on – a target molecule or a target entity. In another aspect the enrichment is conducted by selection for catalytic activity. Alternatively, the conditions enriching the library involve any one or more of electrophoretic separation, gelfiltration, immunoprecipitation, isoelectric focusing, centrifugation, and immobilization.

10

15

The enrichment process can involve cells. Thus, in one embodiment, the conditions enriching the library comprise providing cells capable of internalising the templated molecule, or performing an interaction with the templated molecule having the desired predetermined activity.

20

When the library of complexes have been enriched to a small pool comprising complexes displaying a predetermined activity, it is desirable to obtain each of the complexes separately. The templated molecule can be obtained from the complex by cleaving the linker(s) of the one or more building blocks to release the templated molecule from the template.

25

Nucleotides

The nucleotides used in the present invention may be linked together in an oligonucleotide. Each nucleotide monomer is normally composed of two parts, namely a nucleobase moiety, and a backbone. The back bone may in some cases be subdivided into a sugar moiety and a internucleoside linker.

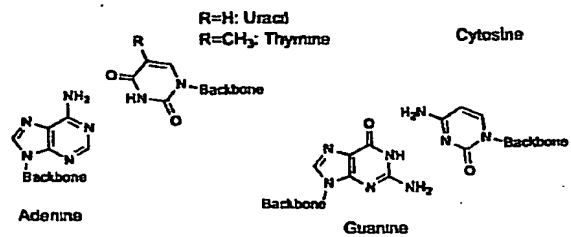
30

The nucleobase moiety may be selected among naturally occurring nucleobases as well as non-naturally occurring nucleobases. It should be clear to the person skilled in the art that various nucleobases which previously have been considered "nonnaturally occurring" have subsequently been found in nature. Thus, "nucleobase" includes not only the known purine and pyrimidine hetero-cycles, but also heterocyclic analogues and tautomers thereof. Illustrative examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, purine, xanthine, diaminopurine, 8-oxo-N⁶-methyladenine, 7-deazaxanthine, 7-deazaguanine, N⁴,N⁴-ethanocytosin, N⁶,N⁶-ethano-2,6-diamino-purine, 5-methylcytosine, 5-(C³-C⁶)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridine, isocytosine, isoguanine, inosine and the "non-naturally occurring" nucleobases described in Benner et al., U.S. Pat No. 5,432,272. The term "nucleobase" is intended to cover every and all of these examples as well as analogues and tautomers thereof. Especially interesting nucleobases are adenine, guanine, thymine, cytosine, 5-methylcytosine, and uracil, which are considered as the naturally occurring nucleobases in relation to therapeutic and diagnostic application in humans.

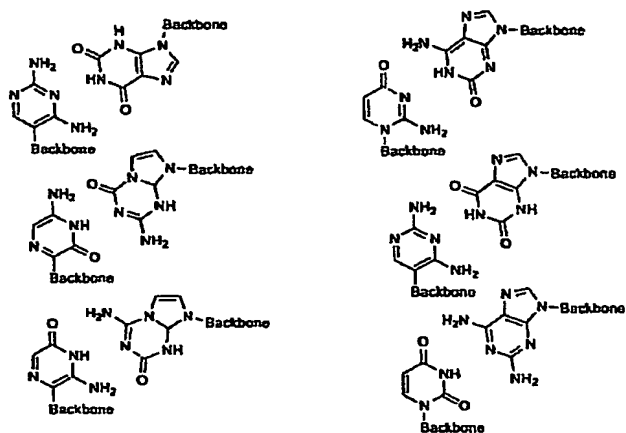
20

Examples of suitable specific pairs of nucleobases are shown below:

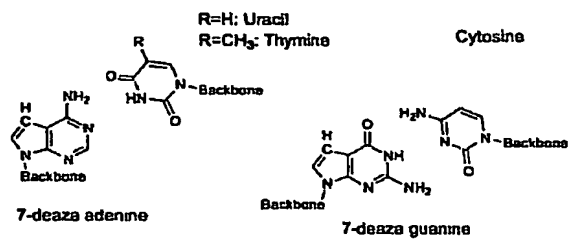
Natural Base Pairs



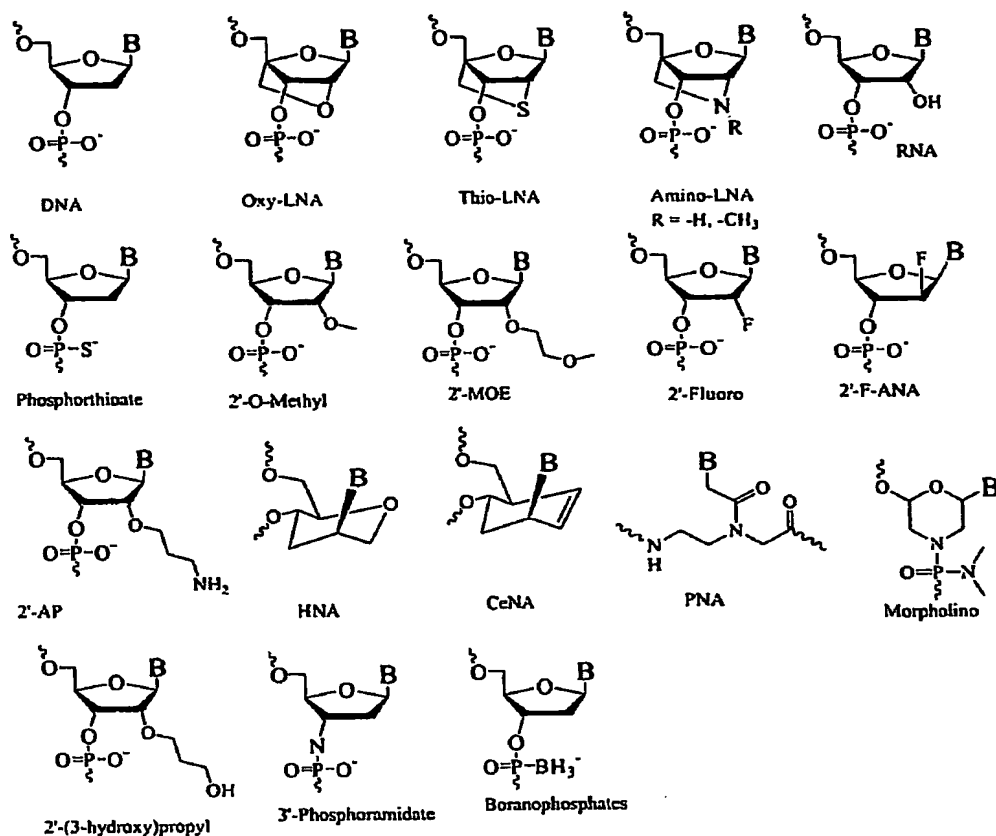
Synthetic Base Pairs



Synthetic purine bases



Suitable examples of backbone units are shown below (B denotes a nucleobase):



- The sugar moiety of the backbone is suitably a pentose but may be the appropriate part of an PNA or a six-member ring. Suitable examples of possible pentoses include ribose, 2'-deoxyribose, 2'-O-methyl-ribose, 2'-fleur-ribose, and 2'-4'-O-methylene-ribose (LNA). Suitably the nucleobase is attached to the 1' position of the pentose entity.
- 5
- 10 An internucleoside linker connects the 3' end of preceding monomer to a 5' end of a succeeding monomer when the sugar moiety of the backbone is a pentose, like ribose or 2'-deoxyribose. The internucleoside linkage may be the natural occurring phosphodiester linkage or a derivative thereof. Examples of such derivatives include phosphorothioate, methylphosphonate, phosphoramidate, phosphotriester, and phosphodithioate. Furthermore, the
- 15

internucleoside linker can be any of a number of non-phosphorous-containing linkers known in the art.

5 Preferred nucleic acid monomers include naturally occurring nucleosides forming part of the DNA as well as the RNA family connected through phosphodiester linkages. The members of the DNA family include deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. The members of the RNA family include adenosine, guanosine, uridine, cytidine, and inosine. Inosine is a non-specific pairing nucleoside and may be used as
10 universal base as discussed above because inosine can pair nearly isoenergetically with A, T, and C.

Each codon is complemented by an anti-codon. The anticodon has the ability specifically to engage with the codon which it complements. The affinity between the codon and the complementing anti-codon is affected through hydrogen bondings following the well-known Watson-Crick base pairing system.
15 Thus, the anti-codon may be composed of the same kind of nucleic acid monomers as the codon itself.

20 Functional groups

The functional entity may comprise one or more functional groups, i.e. groups which eventually form part of the templated molecule. The templated molecule may comprise one or more of the following functional groups either alone or in combination:

- 25
1. Hydroxyls
 2. Primary, secondary, tertiary amines
 3. Carboxylic acids
 4. Phosphates, phosphonates
 5. Sulfonates, sulfonamides

30

 6. Amides
 7. Carbamates
 8. Carbonates

- 9. Ureas
- 10. Alkanes, Alkenes, Alkynes
- 11. Anhydrides
- 12. Ketones
- 5 13. Aldehydes
- 14. Nitratates, nitrites
- 15. Imines
- 16. Phenyl and other aromatic groups
- 17. Pyridines, pyrimidines, purines, indole, imidazole, and heterocyclic
10 bases
- 18. Heterocycles
- 19. polycycles
- 20. Flavins
- 21. Halides
- 15 22. Metals
- 23. Chelates
- 24. Mechanism based inhibitors
- 25. Small molecule catalysts
- 26. Dextrins, saccharides
- 20 27. Fluorescein, Rhodamine and other fluorophores
- 28. Polyketides, peptides, various polymers
- 29. Enzymes and ribozymes and other biological catalysts
- 30. Functional groups for post-polymerization/post activation coupling of
functional groups
- 25 31. Drugs, e.g., taxol moiety, acyclovir moiety, "natural products"
- 32. Supramolecular structures, e.g. nanoclusters
- 33. Lipids
- 34. Oligonucleotides, oligonucleotide analogs (e.g., PNA, LNA, mor-
pholinos)
- 30 35. Hydrogen

Reactive groups

Reactive groups relates among other things to groups which form part of the functional entity and are capable of participating in a reaction that form a connection, either directly or via a suitable bridging molecular entity. Exam-

5 ples of reactive groups are listed below:

1. N-carboxyanhydrides (NCA)
2. N-thiocarboxyanhydrides (NTA)
3. Amines
4. Carboxylic acids
- 10 5. Ketones
6. Aldehydes
7. Hydroxyls
8. Thiols
9. Esters
- 15 10. Thioesters
11. conjugated system of double bonds
12. Alkyl halides
13. Hydrazines
14. N-hydroxysuccinimide esters
- 20 15. Epoxides
16. Haloacetyls
17. UDP-activated saccharides
18. Sulfides
19. Cyanates
- 25 20. Carbonylimidazole
21. Thiazinanones
22. Phosphines
23. Hydroxylamines
24. Sulfonates
- 30 25. Activated nucleotides
26. Vinylchloride
27. Alkenes, quinines

Examples of reactions which the reactive groups can take part in are shown in Fig. 8. Further reactive groups are shown as well in Fig. 8.

5 Templated molecules

According to the present invention, virtually any molecule may be templated using the general method disclosed herein. Examples of compounds which it is anticipated can be synthesised includes, but are not limited to, the compounds listed below:

10

alpha-, beta-, gamma-, and omega-peptides; mono-, di- and tri-substituted peptides; L- and D-form peptides; Cyclohexane- and cyclopentane-backbone modified beta-peptides; Vinylogous polypeptides; glycopolypeptides; polyamides; vinylogous sulfonamide peptide; polysulfonamide; conjugated peptide
 15 (i.e., having prosthetic groups); polyesters; polysaccharides; polycarbamates; polycarbonates; polyureas; poly-peptidylphosphonates; azatides; peptoids (oligo N-substituted glycines); polyethers; ethoxyformacetal oligomers; polythioethers; polyethylene glycols (PEG); polyethylenes; polydisulfides; polyarylene sulfides; polynucleotides; PNAs; LNAs; morpholinos; oligo pyrrolidone;
 20 none; polyoximes; polyimines; polyethyleneimine; polyacetates; polystyrenes; polyacetylene; polyvinyl; lipids; phospholipids; glycolipids; polycycles (aliphatic); polycycles (aromatic); polyheterocycles; proteoglycan; polysiloxanes; polyisocyanides; polyisocyanates; polymethacrylates; monofunctional, Difunctional, Trifunctional and Oligofunctional open-chain hydrocarbons; monofunctional,
 25 difunctional, trifunctional and oligofunctional nonaromatic carbocycles; monocyclic, bicyclic, tricyclic and polycyclic hydrocarbons; bridged polycyclic hydrocarbons; monofunctional, difunctional, trifunctional, and oligofunctional nonaromatic heterocycles; monocyclic, bicyclic, tricyclic, and polycyclic heterocycles, bridged polycyclic heterocycles; monofunctional, difunctional,
 30 trifunctional and oligofunctional aromatic carbocycles; monocyclic, bicyclic, tricyclic, and polycyclic aromatic carbocycles; monofunctional, difunctional, trifunctional and oligofunctional aromatic heterocycles; monocyclic, bicyclic,

tricyclic and polycyclic heterocycles; chelates; fullerenes; steroids; cyclosporin analogs; as well as any combination of the above molecular moieties.

5 Enrichment

Selection or screening, commonly referred to as enrichment, of the library of complexes comprising templated molecules with respect to desired activities (for example binding to particular target, catalytic activity, or a particular effect in an activity assay) may be performed according to any standard protocol. For example, affinity selections may be performed according to the principles used for phage displayed, polysome-displayed or mRNA-protein fusion displayed peptides. Selection for catalytic activity may be performed by affinity selections on transition-state analogue affinity columns (Baca et al. , Proc. Natl. Acad. Sci USA. 1997; 94(19):10063-8), or by function-based selection schemes (Pedersen et al., Proc. Natl. Acad. Sci. USA. 1998, 95(18):10523-8). Screening for a desired characteristic may be performed according to standard microtiter plate-based assays, or by FACS-sorting assays.

Generally, affinity selections involve the immobilisation of a target or a binding partner on a solid support, such as a column. Subsequently, the complex manufactured according to the invention is added to the column under conditions allowing a part of the complexes to bind to the target. The complexes not bound to the target is eluted out of the column and discharged. The part of the complexes attached to the target may be amplified using the template associated with the templated molecule.

The choice of amplification method depends on the choice of codons and anti-codons. Natural oligonucleotides can be amplified by any state of the art method. These methods include, but is not limited to the polymerase chain reaction (PCR); as wells as e.g. nucleic acid sequence-based amplification (e.g. Compton, Nature 350, 91-92 (1991)), amplified anti-sense RNA (e.g. van Gelder et al., PNAS 85: 77652-77656 (1988)); self-sustained sequence

replication system (e.g. Gnatelli et al., PNAS 87: 1874-1878 (1990)); polymerase independent amplification as described in e.g. Schmidt et al., NAR 25: 4797-4802 (1997), as well as in vivo amplification of plasmids carrying cloned DNA fragments. Ligase-mediated amplification methods may
5 also be used, e.g., LCR (Ligase Chain Reaction).

For non-natural nucleotides the choices of efficient amplification procedures are fewer. As non-natural nucleotides per definition can be incorporated by certain enzymes including polymerases, it will be possible to perform manual
10 polymerase chain reaction by adding the polymerase during each extension cycle.

For oligonucleotides containing nucleotide analogs, fewer methods for amplification exist. One may use non-enzyme mediated amplification
15 schemes (Schmidt et al., NAR 25: 4797-4802 (1997)). For backbone-modified oligonucleotide analogs such as PNA and LNA, this amplification method may be used. Before or during amplification the templates or complementing templates may be mutagenized or recombined in order to create a larger diversity for the next round of selection or screening.

20 Following the amplification of the template part of the complex, the method according to the invention is conducted using the amplification product as the templates. The result is a reduced or enriched library of complexes of a template attached to a template molecule.

25 The selection and amplification steps may be repeated if considered necessary to further enrich the library. When the selection and amplification steps are repeated, the binding step involving the target and the complexes, is preferably performed under more strict conditions ensuring that only a part of
30 the complexes adhere to the target.

The enrichment cycles may be performed 2 to 15 times or even more with enrichment in each cycle of 10 to 1000 times. In one approach, the starting library amounts to 10^{14} complexes. After seven cycles of enrichments with a 100 fold concentration in each cycle, the complex with the highest affinity to the target should, theoretically, be obtained. However, it is more likely that the final cycles deliver a small pool of interesting complexes, which have to be examined by other means.

After the final round of selection, it is often desirable to sequence individual templates, in order to determine the composition of individual templated molecules. If the template contains natural nucleotides, it is a standard routine to optionally PCR amplify the isolated templates (if the template is an RNA molecule, it is necessary to use reverse transcriptase to produce cDNA prior to the PCR-amplification), and then clone the DNA fragments into for example plasmids, transform these and then sequence individual plasmid-clones containing one or multiple tandem DNA sequences. In this case, it is practical to design a restriction site in both of the flanking sequences to the central coding sequence of the template (i.e., in the primer binding sites). This will allow easy cloning of the isolated nucleotides. Sequencing can be done by the standard dideoxy chain termination method, or by more classical means such as Maxam-Gilbert sequencing.

If the template contains non-natural nucleotides, it may not be feasible to clone individual sequences by transfer through a microbial host. However, using bead populations where each bead carries one oligonucleotide sequence, it is possible to clone in vitro, where after all the nucleotides attached to a specific bead may be optionally amplified and then sequenced (Brenner et al., 2000, Proc. Natl. Acad. Sci. USA 97, 1665-1670). Alternatively, one may dilute the population of isolates adequately, and then aliquot into micro-titer plates so that the wells on average contain for example 0.1 templates. By amplifying the single templates by for example PCR, it will now be possible to sequence using standard methods. Of course, this requires that the

non-natural nucleotides are substrates for the thermostable polymerase used in the PCR.

5 If alternative methods are used that require shorter oligonucleotides it may be desirable to design the starting template so as to contain restriction sites on either side of the encoding/templating region of the template. Thereby, after the final selection round, the templates can be restricted, to obtain a short oligonucleotide encoding the templated molecule, and then these short oligonucleotides can be applied to various analytical procedures.

10 It is also possible to sequence the isolates by the use of a DNA array of oligonucleotides with random but predetermined sequences.

15 It may also be desirable to sequence the population of isolates as a pool, for example if the sequences are expected to be in register, for example because the initial library consisted of a degenerate sequence based on a polymer sequence with a known (relatively high) desired activity. Therefore, it is then expected that all the isolates have sequences similar to the initial sequence of the templates before selection. Thus, the population of isolates
20 can be sequenced as a whole, to obtain a consensus sequence for the population as a whole.

The present invention is also directed to approaches that allow selection of small molecules capable of binding to different targets. The template-
25 displaying molecule technology contains a built-in function for direct selection and amplification. The binding of the selected molecule should be selective in that they only coordinate to a specific target and thereby prevent or induce a specific biological effect. Ultimately, these binding molecules should be possible to use e.g. as therapeutic agents, or as diagnostic agents.

30 Template-displaying molecule libraries can easily be combined with screenings, selections, or assays to assess the effect of binding of a molecule

- ligand on the function of the target. In a more specific embodiment, the template-displaying method provides a rapid means for isolating and identifying molecule ligands which bind to supra-molecular, macro-supra-molecular, macro-molecular and low-molecular structures (e.g. nucleic acids and proteins, including enzymes, receptors, antibodies, and glycoproteins); signal molecules (e.g. cAMP, inositol triphosphate, peptides, prostaglandins); and surfaces (e.g. metal, plastic, composite, glass, ceramics, rubber, skin, tissue).
- Specifically, selection or partitioning in this context means any process whereby the template-displaying molecule complex bound to a target molecule, i.e. the complex-target pair, can be separated from template-displaying molecules not bound to the target molecule. Selection can be accomplished by various methods known in the art.
- The selection strategy can be carried out so it allows selection against almost any target. Importantly, no steps in this selection strategy need any detailed structural information of the target or the molecules in the libraries. The entire process is driven by the binding affinity involved in the specific recognition/coordination of the molecules in the library to a given target. However, in some applications, if needed, functionality can also be included analogous to selection for catalytic activity using phage display (Soumillion et al. (1994) J. Mol. Biol. 237: 415-22; Pedersen et al. (1998) PNAS. 18: 10523-10528). Example of various selection procedures are described below.
- This built-in template-displaying molecule selection process is well suited for optimizations, where the selection steps are made in series starting with the selection of binding molecules and ends with the optimized binding molecule. The single procedures in each step are possible to automate using various robotic systems. This is because there is a sequential flow of events and where each event can be performed separately. In a most preferable setting, a suitable template-displaying molecule library and the target molecule are

supplied to a fully automatic system which finally generates the optimized binding molecule. Even more preferably, this process should run without any need of external work outside the robotic system during the entire procedure.

- 5 The libraries of template-displayed molecules will contain molecules that could potentially coordinate to any known or unknown target. The region of binding on a target could be into a catalytic site of an enzyme, a binding pocket on a receptor (e.g. GPCR), a protein surface area involved in protein-protein interaction (especially a hot-spot region), and a specific site on DNA
- 10 (e.g. the major groove). The template-displaying molecule technology will primarily identify molecules that coordinate to the target molecule. The natural function of the target could either be stimulated (agonized) or reduced (antagonized) or be unaffected by the binding of the template-displaying molecules. This will be dependent on the precise binding mode and the particular binding-site the template-displaying molecules occupy on the target.
- 15 However, it is known that functional sites (e.g. protein-protein interaction or catalytic sites) on different proteins are more prone to bind molecules that other more neutral surface areas on a protein. In addition, these functional sites normally contain a smaller region that seems to be primarily responsible
- 20 for the binding energy, the so called hot-spot regions (Wells, et al. (1993) Recent Prog. Hormone Res. 48; 253-262). This phenomenon will increase the possibility to directly select for small molecules that will affect the biological function of a certain target.
- 25 The template-displaying molecule technology of the invention will permit selection procedures analogous to other display methods such as phage display (Smith (1985) Science 228: 1315-1317). Phage display selection has been used successfully on peptides (Wells & Lowman. (1992) Curr. Op. Struct. Biol. 2, 597-604) proteins (Marks et al. (1992) J. Biol. Chem. 267:
- 30 16007-16010) and antibodies (Winter et al. (1994) Annu. Rev. Immunol. 12: 433-455). Similar selection procedures are also exploited for other types of display systems such as ribosome display (Mattheakis et al. (1994) Proc.

Natl. Acad. Sci. 91: 9022-9026) and mRNA display (Roberts, et al. (1997) Proc. Natl. Acad. Sci. 94: 12297-302).

5 The linkage between the templated molecule (displayed molecule) and DNA replication unit (coding template) allows an identification of binding molecules using various selection strategies. This invention allows a broad strategy in identifying binding molecules against essentially any known target. In addition, this technology will also allow discovery of novel unknown targets by isolating binding molecules against unknown antigens (epitopes) and use
10 these binding molecules for identification and validation.

As will be understood, selection of binding molecules from the template-displaying molecule libraries can be performed in any format to identify optimal binding molecules. A typical selection procedure against a purified target
15 will include the following major steps: Generation of a template-displaying molecule library; Immobilization of the target molecule using a suitable immobilization approach; Adding the library to allow binding of the template-displayed molecules; Removing of the non-binding template-displayed molecules; Elution of the template-displayed molecules bound to the immobilized
20 target; Amplification of enriched template-displaying molecules for identification by sequencing or to input for the next round of selection. The general steps are schematically shown in Figure 12.

In a preferred embodiment, a standard selection protocol using a template-displaying molecule library is to use the bio-panning method. In this technique, the target (e.g. protein or peptide conjugate) is immobilized onto a solid support and the template-displayed molecules that potentially coordinate to the target are the ones that are selected and enriched. However, the selection procedure requires that the bound template-displayed molecules
25 can be separated from the unbound ones, i.e. those in solution. There are many ways in which this might be accomplished as known to ordinary skilled in the art.
30

The first step in the affinity enrichment cycle (one round as described in Figure 12) is when the template-displayed molecules showing low affinity for an immobilized target are washed away, leaving the strongly binding template-displayed molecules attached to the target. The enriched population, remaining bound to the target after the stringent washing, is then eluted with, e.g. acid, chaotropic salts, heat, competitive elution with the known ligand or proteolytic release of the target/template molecules. The eluted template-displayed molecules are suitable for PCR, leading to many orders of amplification, i.e. every single template-displayed molecule enriched in the first selection round participates in the further rounds of selection at a greatly increased copy number. After typically three to ten rounds of enrichment a population of molecules is obtained which is greatly enriched for the template-displayed molecules which bind most strongly to the target. This is followed quantitatively by assaying the proportion of template-displaying molecules which remain bound to the immobilized target. The variant template sequences are then individually sequenced.

Immobilisation of the target (peptide, protein, DNA or other antigen) on beads might be useful where there is doubt that the target will adsorb to the tube (e.g. unfolded targets eluted from SDS-PAGE gels). The derivatised beads can then be used to select from the template-displaying molecules, simply by sedimenting the beads in a bench centrifuge. Alternatively, the beads can be used to make an affinity column and the template-displaying libraries suspension recirculated through the column. There are many reactive matrices available for immobilizing the target molecule, including for instance attachment to $-NH_2$ groups and $-SH$ groups. Magnetic beads are essentially a variant on the above; the target is attached to magnetic beads which are then used in the selection. Activated beads are available with attachment sites for $-NH_2$ or $-COOH$ groups (which can be used for coupling). The target can be also be blotted onto nitrocellulose or PVDF. When using a blotting strategy, it

is important to make sure the strip of blot used is blocked after immobilization of the target (e.g. with BSA or similar protein).

In another preferred embodiment, the selection or partitioning can also be performed using for example: Immunoprecipitation or indirect immunoprecipitation where the target molecule is captured together with template-displaying binding molecules; affinity column chromatography where the target is immobilized on a column and the template-displaying libraries are flowed through to capture target-binding molecules; gel-shift (agarose or polyacrylamide) where the selected template-displaying molecules migrate together with the target in the gel; FACS sorting to localize cells that coordinates template-displaying molecules; CsCl gradient centrifugation to isolate the target molecule together template-displaying binding molecules; Mass spectroscopy to identify target molecules which are labelled with template-displaying molecules; etc., without limitation. In general, any method where the template-displaying molecule/target complex can be separated from template-displaying molecules not bound to the target is useful.

Table 1: Examples of selection method possible to use to identify binding molecules using the template-displaying technology.

Type of Target	Method of choice
Soluble receptors	Direct immobilization, Immunoprecipitation, affinity column, FACS sorting, MS.
Cell surface receptor	Cell-surface subtraction selection, FACS sorting, Affinity column.
Enzyme inhibitors	Direct immobilization, Immunoprecipitation, affinity column, FACS sorting, MS.
Surface epitopes	Cell-surface subtraction selection, in-vivo selection, FACS sorting, Affinity column.

Elution of template-displayed molecules can be performed in different ways. The binding molecules can be released from the target molecule by denaturation, acid, or chaotropic salts and then transferred to another vial for amplification. Alternatively, the elution can be more specific to reduce the background. Elution can be accomplished using proteolysis to cleave a linker between the target and the immobilizing surface or between the displaying molecule and the template. Also, elution can be accomplished by competition with a known ligand. Alternatively, the PCR reaction can be performed directly in the washed wells at the end of the selection reaction.

A possible feature of the invention is the fact that the binding molecules need not be elutable from the target to be selectable since only the encoding template DNA is needed for further amplification or cloning, not the binding molecule itself. It is known that some selection procedure can bind the most avid ligands so tightly as to be very difficult to elute. However the method of the invention can successfully be practiced to yield avid ligands, even covalent binding ligands.

Alternative selection protocol includes a known ligand as fragment of each displayed molecule in the library. That known ligand will guide the selection by coordinate to a defined part on the target molecule and focus the selection to molecules that binds to the same region. This could be especially useful for increasing the affinity for a ligand with a desired biological function but with a too low potency.

A further aspect of the present invention relates to methods of increasing the diversity or complexity of a single or a mixture of selected binding molecules. After the initial selection, the enriched molecules can be altered to further increase the chemical diversity or complexity of the displayed molecules. This can be performed using various methods known to the art. For example, using synthesized randomized oligonucleotides, spiked oligonucleotides or random mutagenesis. The randomization can be focused to allow preferable codons or localized to a predetermined portion or sub-sequence of the tem-

plate nucleotide sequence. Other preferable method is to recombine templates coding for the binding molecules in a similar manner as DNA shuffling is used on homologous genes for proteins (Stemmer (1994) Nature 370:389-91). This approach can be used to recombine initial libraries or more preferably to recombine enriched encoding templates.

In another embodiment of the invention when binding molecules against specific antigens that is only possible to express on a cell surface, e.g. ion channels or transmembrane receptors, is required, the cells particle themselves can be used as the selection agent. In this sort of approach, cells lacking the specific target should be used to do one or more rounds of negative selection or be present in large excess in the selection process. Here, irrelevant template-displayed molecules are removed. For example, for a positive selection against a receptor expressed on whole cells, the negative selection would be against the untransformed cells. This approach is also called subtraction selection and has successfully been used for phage display on antibody libraries (Hoogenboom et al. (1998) Immunotech. 4: 1-20).

A specific example of a selection procedure can involve selection against cell surface receptors that become internalized from the membrane so that the receptor together with the selected binding molecule can make its way into the cell cytoplasm or cell nucleus. Depending on the dissociation rate constant for specific selected binding molecules, these molecules largely reside after uptake in either the cytoplasm or the nucleus.

The skilled person in the art will acknowledge that the selection process can be performed in any setup where the target is used as the bait onto which the template-displaying molecules can coordinate.

The selection methods of the present invention can be combined with secondary selection or screening to identify molecule ligands capable of modifying target molecule function upon binding. Thus, the methods described herein can be employed to isolate or produce binding molecules which bind to and

modify the function of any protein or nucleic acid. It is contemplated that the method of the present invention can be employed to identify, isolate or produce binding molecules which will affect catalytic activity of target enzymes, *i.e.*, inhibit catalysis or modifying substrate binding, affect the functionality of protein receptors, *i.e.*, inhibit binding to receptors or modify the specificity of binding to receptors; affect the formation of protein multimers, *i.e.*, disrupt quaternary structure of protein subunits; and modify transport properties of protein, *i.e.*, disrupt transport of small molecules or ions by proteins.

10 A still further aspect of the present invention relates to methods allowing functionality in the selection process can also be included. For example, when enrichment against a certain target have been performed generation a number of different hits, these hits can then directly be tested for functionality (*e.g.* cell signalling). This can for example be performed using fluorescence-activated cell sorting (FACS).

15 The altered phenotype may be detected in a wide variety of ways. Generally, the changed phenotype is detected using, for example: microscopic analysis of cell morphology; standard cell viability assays, including both increased cell death and increased cell viability; standard labelling assays such as fluorometric indicator assays for the presence of level of particular cell or molecule, including FACS or other dye staining techniques; biochemical detection of the expression of target compounds after killing the cells; etc. In some cases, specific signalling pathways can be probed using various reporter gene constructs.

20 Secondary selection methods that can be combined with template-displaying molecule technology include among others selections or screens for enzyme inhibition, alteration or substrate binding, loss of functionality, disruption of structure, etc. Those of ordinary skill in the art are able to select among various alternatives of selection or screening methods that are compatible with the methods described herein.

The binding molecules of the invention can be selected for other properties in addition to binding. For example, during selection; stability to certain conditions of the desired working environment of the end product can be included as a selection criterion. If binding molecules which are stable in the presence of a certain protease is desired, that protease can be part of the buffer medium used during selection. Similarly, the selection can also be performed in serum or cell extracts or any type of media. As will be understood, when utilizing this template-displaying approach, conditions which disrupt or degrade the template should be avoided to allow amplification. Other desired properties can be incorporated, directly into the displaying molecules as will be understood by those skilled in the art. For example, membrane affinity can be included as a property by employing building blocks with high hydrophobicity.

Molecules selected by the template-displaying molecule technology can be produced by various synthetic methods. Chemical synthesis can be accomplished since the structure of selected binding molecules is readily obtained from the nucleic acid sequence of the coding template. Chemical synthesis of the selected molecules is also possible because the building blocks that compose the binding molecules are also known in addition to the chemical reactions that assemble them together.

In a preferred embodiment, the selected binding molecules is synthesized and tested in various appropriate *in vitro* and *in vivo* testing to verify the selected candidates for biological effects and potency. This may be done in a variety of ways, as will be appreciated by those in the art, and may depend on the composition of the bioactive molecule.

Target identification and validation

In another aspect, the present invention provides methods to identify or isolate targets that are involved in pathological processes or other biological events. In this aspect, the target molecules are again preferably proteins or

nucleic acids, but can also include, among others, carbohydrates and various molecules to which specific molecule ligand binding can be achieved. In principal, the template-displaying molecule technology could be used to select for specific epitopes on antigens found on cells, tissues or *in vivo*. These epitopes might belong to a target that is involved in important biological events. In addition, these epitopes might also be involved in the biological function of the target.

Phage display with antibodies and peptide libraries has been used numerous times successfully in identifying new cellular antigens. (e.g. Pasqualini et al. (1996) *Nature* 380: 364-366; Pasqualini et al. (2000) *Cancer Res.* 60: 722-727; Scheffer et al. (2002) *Br J Cancer* 86: 954-962; Kupsch et al. (1999) *Clin Cancer Res.* 5: 925-931; Tseng-Law et al. (1999) *Exp. Hematol.* 27: 936-945; Gevorkian et al. (1998) *Clin. Immunol. Immunopathol.* 86: 305-309). Especially effective have been selection directly on cells suspected to express cell-specific antigens. Importantly, when selecting for cell-surface antigen, the template molecule can be maintained outside the cell. This will increase the probability that the template molecule will be intact after release for the cell surface.

In vivo selection of template-displayed molecules has tremendous potential. By selecting from libraries of template-displayed molecules *in vivo* it is possible to isolate molecules capable of homing specifically to normal tissues and other pathological tissues (e.g. tumours). This principle has been illustrated using phage display of peptide libraries (Pasqualini & Ruoslahti (1996) *Nature* 380: 364-366). This system has also been used in humans to identify peptide motifs that localized to different organs (Arap et al. (2002) *Nat. Med.* 2:121-127). A similar selection procedure could be used for the template-displaying libraries. The coding DNA in phage display protected effectively by the phage particle allows selection *in vivo*. Accordingly, the stability of the template *in vivo* will be important for amplification and identification. The template can be stabilised using various nucleotide derivatives in a similar

way as have been used to stabilise aptamers for in vivo applications (Nolte (1996) *Nature Biotechnol.* 14: 1116-1121; Pagratis et al. (1997) *Nature Biotechnol.* 15: 68-72). However, it is reasonable to believe that the template structure will be stabilized against degradation due to the modified bases used for encoding the displayed molecule. Other types of protection are also possible where the template molecule is shielded for the solution using various methods. This could include for example liposomes, pegylation, binding proteins or other sorts of protection. The template molecule could also be integrated into another designed structure that protects the template from external manipulation. For example, the linker can be designed to be incorporated in vesicles to position the templates inside the vesicle and the displaying molecules on the outside. The arrangement will protect the template molecules from external manipulation and at the same time allow exposure of the displaying molecules to permit selection.

Most antibodies have a large concave binding area which requires to some degree protruding epitopes on the antigens. Also, the antibody molecule is a large macromolecule (150 KDa) which will sterically reduce the access for a number of different antigens (e.g. on a cell surface). The template-displaying technology should be able to access and recognize epitopes inaccessible to antibodies. The small binding molecules will be able to bind into active sites, grooves and other areas on an antigen. The coding template element is also smaller than an antibody which will increase the physical access of the template-binding molecule pair. In addition, the diversity and complexity of the template-displaying molecule libraries will be much greater compared to peptide libraries. This will increase the possibility to find molecules that can coordinate to epitopes inaccessible to peptides due to inadequate chemistry. All together, the template-displaying molecule technology has the potential to identify novel antigens which is not possible to identify with antibodies or peptides. One of ordinary skill in the art will acknowledge that various types of cells can be used in the selection procedure. It will also be understood that

the selection for new antigens can be performed using subtraction methods as described previously.

5 Another aspect of the present invention relates to methods to validate the identified target. The identified binding molecules can directly be used if they change the biological response of the target. This can be done either *in vitro* using any direct or cell-based assay or directly *in vivo* studying any phenotypic response. The strength of this approach is that the same molecules are used both for identification and validation of various targets. Most favourable,
10 the binding molecules could also directly be used as therapeutic agents.

In another preferred embodiment, the template-displaying molecules are used to pull out the target molecules. This can for instance be achieved by selection against a cDNA library expressed on bacteriophage (libraries vs.
15 libraries). By mixing a template-displaying molecule library with a cDNA library it will be possible to find binding pairs between the small molecules in the template-displaying molecule library and proteins from the cDNA library. One possibility is to mix a phage display library with a template display library and do a selection for either the phage or template library. The selected li-
20 brary is then plated to localized phage clones and the DNA coding for the phage and template displayed molecules can then be identified using PCR. Other types of libraries than cDNA could also be used such as nucleic acids, carbohydrates, synthetic polymer.

25 In another embodiment of the invention the template-displaying molecule technology can be used to account for *in vivo* and *in vitro* drug metabolism. That could include both phase I (activation) and phase II (detoxification) reactions. The major classes of reactions are oxidation, reduction, and hydrolysis. Other enzymes catalyze conjugations. These enzymes could be used as tar-
30 gets in a selection process to eliminate displayed molecule that are prone to coordinate to these enzymes. The templates corresponding to these displayed molecules could subsequently be used to compete or eliminate these

molecules when making template-displaying molecule libraries. These obtained libraries will then be free of molecules that will have a tendency of binding to enzymes involved in phase I-II and possible be faster eliminated. For instance, a selection on each separate enzyme or any combination of cytochrome P450 enzymes, flavin monooxygenase, monoamine oxidase, esterases, amidases, hydrolases, reductases, dehydrogenases, oxidases UDP-glucuronosyltransferases, glutathione S-transferases as well as other relevant enzymes could be performed to identify these binding molecules that are prone to coordinate to these metabolic enzymes. Inhibitors are easily selected for due to their binding affinity but substrates need at least micro molar affinity to be identified.

Another interesting embodiment of this invention is the possibility to directly select for molecules that passively or actively becomes transported across epithelial plasma membrane, or other membranes. One possible selection assay is to use CaCO-2 cells, a human colon epithelial cell line, which is general, accepted as a good model for the epithelial barrier in the gastrointestinal guts. The CaCO-2 assay involves growing a human colon epithelial cell line on tissue culture well inserts, such that the resultant monolayer forms a biological barrier between apical and basolateral compartments. The template-displaying molecule libraries are placed either side of the cell monolayer and the molecules that can permeate the cell monolayer is collected and amplified. This process can be repeated until active molecules have been identified. Other cell line or setup of this assay is possible and is obvious for skill in the art.

A still further aspect of the present invention relates methods of selecting for stability of the selected molecules. This could be performed by subjecting an enriched pool of binding molecules to an environment that will possibly degrade or change the structure of the binding molecules. Various conditions could be certain proteases or a mixture of protease, cell extract, and various fluids from for example the gastrointestinal gut. Other conditions could be

various salts or acid milieu or elevated temperature. Another possibility is to generate a library of known ligands and subject that library to stability tests and selection to identify stable molecules under certain conditions as describe above.

5

Therapeutic applications

The template-displaying molecule technology of the invention may be used for blocking or stimulating various targets. A therapeutically relevant target is a substance that is known or suspected to be involved in a regulating process that is malfunctioning and thus leads to a disease state. Examples of such processes are receptor-ligand interaction, transcription-DNA interaction, and cell-cell interaction involving adhesion molecules, cofactor-enzyme interaction, and protein-protein interaction in intracellular signalling. Target molecule means any compound of interest for which a molecule ligand is desired. Thus, target can, for example, include a chemical compound, a mixture of chemical compounds, an array of spatially localized compounds, a biological macromolecule, such as DNA or mRNA, a bacteriophage peptide display library, a ribosome peptide display library, an extract made from biological materials such as bacteria, plants, fungi, or animal (e.g. mammalian) cells or tissue, protein, fusion protein, peptide, enzyme, receptor, receptor ligand, hormone, antigen, antibody, drug, dye, growth factor, lipid, substrate, toxin, virus, or the like etc., without limitation. Other examples of targets include, e.g. a whole cell, a whole tissue, a mixture of related or unrelated proteins, a mixture of viruses or bacterial strains or the like. etc., without limitation.

25

Therapeutic drug targets can be divided into different classes according to function; receptors, enzymes, hormones, transcription factors, ion channels, nuclear receptors, DNA, (Drews, J. (2000) Science 287:1960-1964). Among those, receptors, nuclear receptors, and metabolic enzymes constitute overwhelmingly the majority of known targets for existing drugs. Especially, G Protein-Coupled Receptors (GPCR) constitutes one of the most important classes of drug targets together with proteases for pharmacological interven-

30

tion. Although the above examples are focused on the most relevant targets, it will be self-evident for a person skilled in the art that any other therapeutic target may be of interest.

5 The present invention employing the template-displaying molecule technology can be utilized to identify agonists or antagonists for all these classes of drug targets, dependent on the specific properties each target holds. Most of the targets are possible to obtain in a purified form for direct selection procedures. Other targets have to be used when they are in their native environments such as imbedded cell surface receptors. In those situations the selection using the template-displaying molecule libraries can be performed using subtraction-selection described previously.

15 One specific application of the template-displaying molecule technology of the invention is to generate molecules that can function as antagonists, where the molecules block the interaction between a receptor and one or more ligands. Another application includes cell targeting. For example, the generated molecules recognizing specific surface proteins or receptors will be able to bind to certain cell types. Such molecules may in addition carry another therapeutic agent to increase the potency and reduce the side-effects (for example cancer treatment). Applications involving antiviral agents are also included. For example, a generated molecule, which binds strongly to epitopes on the virus particle, may be useful as an antiviral agent.

25 Another specific application of the template-displaying molecule technology of the invention is to generate molecules that can function as agonists, where the molecules stimulate or activate a receptor to initiate a cellular signalling pathway.

Brief Description of the Figures

30 Fig. 1 schematically shows templated synthesis of a scaffolded molecule, Fig. 2 schematically shows templated synthesis of a linear molecule covalently attached to the template.

Fig. 3 schematically shows synthesis of a templated scaffolded molecule attached by hybridisation to the template.

Fig. 4 Schematically shows the templated synthesis of a scaffolded molecule using building blocks hybridized to multiple codons.

5 Fig. 5 shows the templated synthesis of a linear molecule, which is attached by hybridisation to the template.

Fig. 6 shows a templated synthesis where the nascent templated molecule changes building blocks during synthesis (zig-zag synthesis).

Fig. 7 shows an example of β -peptide zig-zag synthesis.

10 Fig. 8 shows various examples of reactions resulting in a transfer of functional entity.

Fig. 9 shows pairs of reactive groups X and Y as well as the resulting bond.

Fig. 10 shows examples of the formation of building blocks.

Fig. 11 shows examples of cleavable linkers.

15 Fig. 12 shows a typical panning protocol for selection of template-displaying molecule complexes.

Detailed Description of the Invention

20 In Fig. 1, a template comprising three codons is initially provided. Each codon comprises a sequence of 5 nucleotides selected among A, C, and T. This provides for a ternary system with $3^5 = 243$ different combinations. The codons are spaced with a constant region of GG to provide for affinity. The template is in a first step coupled to a scaffold at the end of the template which contains the first codon. The scaffold shown has three reactive groups (Y), which may all be amine ($-\text{NH}_2$). In a second step, a building block O1 is annealed to the template. The building block comprises the anticodon (A-cdn 1) which complements codon 1 of the template. The anticodon is connected to a functional group R_1 through a reactive group X. The reactive group X is suitably an ester which upon reaction with the reactive group Y (amine) can be cleaved. In a third step the functional entity of the first building block is transferred to the scaffold by a direct reaction involving the reactive groups X and Y. In the event X is amine ($\text{Sca}-\text{NH}_2$) and Y is ester ($R_1-\text{C}(=\text{O})\text{O}-$

25

30

oligonucleotide), the formed connection is an amide ($R_1-C(=O)-NH-Sca$). Subsequent to the transfer of the functional group R_1 to the scaffold the remainder of the first building block is removed from the template by increasing the temperature to a level above the annealing temperature. An alternative to the direct transfer of a functional entity to the scaffold is a two-step procedure, wherein a connection between the functional entity and the scaffold is formed in a first step and the functional entity in a second step is cleaved from the remainder of the building block.

At the single strand state of the template the second building block is added and the temperature is slowly decreased. The second building block comprises an anticodon (A-cdn 2) which complements codon 2 of the template. At the opposite position of the first codon, a sequence of universal bases, such as inosine, is present. The sequence of the universal bases is attached to a functional group R_2 through a reactive group X. Because the second building block anneals to a longer stretch of the template, the annealing temperature is higher for the second building block compared to the first building block. Therefore, the risk for reannealing of the first building block to the template is prevented or significantly reduced at the temperature where the second building block is annealed to the template.

In a fourth step, following the annealing of the second building block to the template, the reaction between the reactive groups X and Y is allowed to occur. The reaction result in a simultaneous formation of connection and cleavage of the linkage between the functional group R_2 and the anticodon part of the building block. For simplicity, the same reactive groups as for the first building block is shown, however, it will be immediately clear to the skilled person that a variety of other reactive groups are available, e.g. as shown in Fig. 8. When the transfer of the functional group to the scaffold has occurred, the remainder of the second building block is removed by increasing the temperature above the annealing temperature for the second building block. Subsequently, the third building block is added to the template.

The third building block comprises the third anticodon, which complements the codon 3 of the template. At the positions opposite codon 1 and codon 2, the third building block is provided with universal bases. The universal bases
5 ensure that the building block will anneal to the template through out the oligonucleotide part of the building block. The third building block will have an annealing temperature which is higher than the second as well as the first building block because the third building block anneals to a larger stretch of the template oligonucleotide. Thus, when the temperature is decreased from
10 a single stranded state of the template only or mainly the third building block will anneal to the template.

In a fifth step the third functional group R_3 is transferred to the scaffold as explained above for R_1 and R_2 . The resulting scaffolded molecule at the end
15 of the template has been encoded by the codons of the template. The template specifies the kind of functional group transferred to the scaffold as well as the order in which the functional groups have been transferred.

A library may be generated based on the above system. As each coding region provides for the generation of 243 different unique codons it is, at least
20 theoretically, possible to encode $243^3 = 14,348,907$ templated molecules. The library may be subjected to a selection procedure with a view to identify one or more candidates for alleviating a disorder. Typically, an affinity assay is conducted. The stringency of the affinity assay may be adjusted to allow
25 only for about 1/100 of the templated molecules to bind to the targets. Following each selection cycle, the selected templates may be amplified using conventional techniques, such as PCR. The amplified templates may be taken through the same method as described above to generate more of the selected templated compound. An important feature of the present invention is
30 that it is not necessary to identify the compounds which binds to the target through out the method. Of course in a final stage, it will be preferred to decipher the codons in order to establish the composition of the templated mole-

cule to make it possible to develop alternative chemical or biological ways of obtaining the target binding molecule.

Fig. 2 shows a method for the generation of a linear templated molecule. Initially a template with three codons in sequence is provided. Each of the codons comprises a sequence of 5 nucleobases selected among A, T, and C and is separated by a constant sequence of two or three G's. In a first step a linker (L) with a reactive group (X) is attached to one end of the template. The linker is selectively cleavable, i.e. is not cleavable during the synthesis of the templated molecule, but can be cleaved subsequent to the generation of the templated molecule. In a second step a first building block is annealed to the template. The first building block (O1) comprises an anticodon (A-cdn 1) complementing the codon 1 of the template. The oligonucleotide comprising the anticodon 1 has, optionally through a constant sequence, attached thereto a functional entity which comprises a reactive group (not shown). The constant sequence can provide for a high affinity at the end of the nucleotides. The arrow of step 2 of the fig. 2 shows the attack of the reactive group of the functional entity toward the template reactive group. In a third step the functional entity R1 is transferred to the template by a reaction involving the reactive group X and the functional entity reactive group. Subsequent to the reaction, the remainder of building block 1 (O1) is removed. Suitably, the removal is conducted by increasing the temperature above the annealing temperature of the nucleotide part of the first building block and the template and the second building block is added. The second building block comprises the second anticodon (A-cdn 2) which is capable of hybridising to codon 2 of the template. The second anticodon is connected to a sequence of universal nucleobases (UB) at a position opposite the first codon. The stretch of universal nucleobases is attached to a second functional entity (R₂), optionally through a constant sequences of nucleotide. Due to the longer region of the second building block compared to the first building block, it will predominately be attached to the template.

In step 4, the second functional entity is transferred to the first functional entity provided on the template in a preceding step. The step presupposes that the first functional entity has a reactive group available for generation of a connection to the second functional entity. After the reaction, the remainder of the second building block is detached from the template codon by increasing the temperature and a third building block (O3) is added. The third building block comprises an anticodon (A-cdn 3) which can bind to the third codon of the template and stretches of universal nucleobases at position opposite the first and the second codon. The stretches of universal nucleobases are coupled to a third functional entity (R_3), optionally through a constant sequence. In a fifth step the R_3 is transferred to R_2 of the template-directed molecule. The remainder of the third building block may be maintained annealed to the template or removed, as desired. In a step not shown the selectively cleavable linker (L) is cleaved to release the template-directed molecule.

A library as described for the embodiment shown in fig. 1 can be created and subjected to selection procedures.

Fig. 3 shows an embodiment in which the scaffold is not attached covalently to the template as disclosed in Fig. 1. In the contrary, the scaffold is attached to an oligonucleotide which in turn is attached by hybridisation to the template. In other words, the reactive group(s) of the scaffold are non-covalently attached to the template. Initially, a template is provided. The template comprises two coding region. In the first coding region, a codon is provided which encode the scaffold. This makes it possible to generate a library in which different scaffolds occur. The other coding region comprises the codons which encode the functional entities which are attached to the scaffold.

In a first step the template is contacted with the building block harbouring the scaffold (O4) and the building block comprising the first functional entity (O1). O4 comprises an anticodon (A-cdn 4) which complements the codon 4 of the

template. The anticodon 4 is furthermore attached to a scaffold comprising three reactive groups (Y). In a preferred aspect, the oligonucleotide part of O4 has an annealing temperature above the annealing temperature of at least the second building block to allow for the scaffold building block to remain bound to the template even during the detachment steps of the reacted first and second building blocks. The first functional entity building block (O1) comprises a first anticodon (A-cdn 1) which complements the codon 1 of the template. The anticodon is attached to a functional entity (-X-R₁) via the reactive group and optionally interspaced by a constant region.

In a second step, the functional entity of the first building block is transferred to the scaffold. After the transfer, the remainder of the first building block is removed by increasing the temperature above the annealing temperature and a second building block (O2) is added. The second building block comprises an anticodon complementing the codon 2 of the template and is connected to a stretch of universal nucleobases complementing the codon 1 of the template. The stretch of universal nucleobases is in turn attached to a functional entity via the reactive group of the functional entity. Optionally a constant region is present between the stretch of universal nucleobases and the functional entity reactive group to provide for a high adherence at the reactive group. In a third step the second functional entity is transferred to the scaffold and the remainder of the second building block is removed by increasing the temperature or, alternatively, by incorporating a biotin moiety in the building block and subjecting to streptavidin bound to a solid surface. Subsequently, the third building block (O3) is added and the temperature is decreased slowly to allow the third building block to anneal to the template. In a fourth step the third functional entity is transferred to the scaffold.

An example illustrating the embodiment shown in fig. 3 is depicted below:

Template:

5'-AGCGCTAACTGAGACNNNNNAGAGHHHHHGGHHHHHGGHHHHHG-
GCTCGACATGCGTA-3'

5 Nucleotide sequence of scaffold building block

3'-TCGCGATTGACTCTGNNNNNT-5'-Sca(Y)₃

(1024 different anti-codons possible)

Nucleotide sequence of functional entity building blocks

10 First building block:

FE₁-CTCDDDDDDCC-5'

Second building block:

FE₂-CTCIIIIICDDDDDDCC-5'

15

Third building block:

FE₃-CTCIIIIICCIIIIICDDDDDDCC-5'

Abbreviations:

20 A = adenosine, C = cytosine, G = guanosine, T = thymidine, I = Inosine

N = A, C, G or T

H = C, A or T

D = A, G or T

FE_n = Functional entity of building block n

25 Y = reactive group of scaffold.

The template comprises, listed from the 5' end:

- a first priming site (AGCGC TAACT GAGAC) which also serves as affinity providing sequence for the scaffold,
- 30 • a codon encoding the scaffold (NNNNN). N may be selected as appropriate among the 4 naturally occurring nucleobases. Thus, in the generation of a library, a total of $4^5 = 1024$ different variations exist.

- an affinity providing sequence for the scaffold building block (A)
- an affinity providing sequence for the functional entity building blocks (GAG)
- 5 • a first building block codon (HHHHH). H may be selected among the three nucleobases C, A, and T. Therefore, the total number of different combinations is $3^5 = 243$.
- an affinity providing sequence (GG)
- 10 • a second building block codon (HHHHH). H may be selected among the three nucleobases C, A, and T. Therefore, the total number of different combinations is $3^5 = 243$.
- An affinity providing sequence (GG)
- A third building block codon (HHHHH). H may be selected among the three nucleobases C, A, and T. Therefore, the total number of different combinations is $3^5 = 243$.
- 15 • An affinity providing sequence (GG)
- A second priming site (GGCTCGACATGCGTA).

20 The total number of combinations is more than 10^{10} . Some of the combinations are only of theoretical interest, e.g. because the template will tend to self-hybridise. However, a sufficient number of combinations remain to allow the experimenter to design a library of a considerable size.

25 The reactive group of the building blocks are attached to a constant region which provide for a high affinity between the template and the building block in the vicinity of the reactive group. The constant region is followed by anti-codon region 1. The set of building blocks having anti-codons which hybridise to codons in codon region 1 is said to have the reactive group proximal to the anticodon. The anti-codon region is followed by a constant section (CC) which ensures annealing in frame. The second set of building blocks has anti-codons which anneal specifically to codons of the codon region 2. The second set of building blocks comprises a sequence of inosine at the position

30 opposite to the first codon sequence and the third set of building blocks com-

prise a sequence of inosine at the position opposite the first as well as the second codon sequences. The sets of building blocks in which the anticodon and the functional entity is interspaced by one or more sequences of a universal nucleobase such as inosine, is said to have the anticodon placed distal to the template reactive group.

The library is generated by, in a first step, to contacting the plurality of templates with the set of scaffold building blocks and the set of building block having anticodons which specifically anneals to the first codon region. The addition of the building blocks to the templates is conducted at a temperature in which all the constituent are single stranded. The temperature is then slowly decreased to allow the individual codon:anticodon hybrids to be formed. After the annealing of the two sets of building blocks to the template, the reactive group of the template is delivered a functional entity by a reaction involving simultaneous connection and cleavage. The remaining parts of the first set of building blocks are removed by increasing the temperature above the annealing temperature and a second set of building blocks are added. The temperature is decreased below the annealing temperature of the second set of building blocks and a transfer of the second functional entity are effected. Subsequently, the remainder of the second set of building blocks is removed and the third set of building blocks are added and annealed to the plurality of templates.

The resulting template-directed library of molecules can be subjected to a selection procedure as described for the embodiment according to Fig. 1. Following each selection round the selected subset of templates are amplified by PCR using the priming sites of the templates for the annealing of primers.

Fig. 4 shows an embodiment similar to the embodiment of Fig. 3, however with the difference that the building blocks are provided with an oligonucleotide having essentially the same annealing temperature. Following each

transfer the spent building block is removed selectively. A preferred method is to provide the oligonucleotide with a biotin moiety during the synthesis of the oligonucleotide. Streptavidin has an ability to bind to biotin, which may be used for separating the spent building block from the template. If the streptavidin is coupled to the surface of beads, the spent building blocks can be removed by mixing the template:building block hybrid with streptavidin coated beads at conditions which allow the binding of biotin to streptavidin; increasing the stringency to separate the template from the building block; and subjecting the mixture to a gravity force, e.g. microspinning, to separate the beads carrying the spent building blocks from the template. Streptavidin may also be coupled to the surface of a column matrix. The spent building blocks can be separated from the template by pouring the mixture comprising the template:building block hybrid into a column comprising a streptavidin-containing matrix material under conditions which allow streptavidin to bind to biotin. Subsequently, the stringency is increased, e.g. by increasing the temperature to a level at which the annealing between the template and the spent building block is ruptured. Next, the template is eluted out of the column with a suitable buffer while the spent building block remains bound to the matrix material.

20

Fig. 5 shows an embodiment in which a linear molecule is formed. Compared to the Fig. 3, differences in the building blocks as well as the product produced can be observed. The reactive group of the template (X) is attached through a selectively cleavable linker (L) to the anticodon 4 (A-cdn 4). Anticodon 4 complements codon 4 of the template and provides for attachment of the reactive group to the template. The building blocks used in the present embodiment comprise a functional entity R_n which have dual properties of being transferred to the template reactive group X or a nascent template-directed molecule as well as being capable of receiving in the next transferred functional entity. This requires either the presence of two reactive groups capable of participating in the formation of a connection or a single reactive group which upon transfer is transformed to another reactive group.

25

30

In a first step, the complementing element comprising the anticodon 4 and the first building block are added to the template under annealing conditions. In step 2, the first functional entity R_1 is transferred to the complementing element by a reaction involving the reactive group X. Subsequently, the re-
5 remained of the first building block is removed and a second building block is added under selective hybridisation conditions. Step 3 starts with a transfer of the functional entity R_2 to the previous transferred R_1 or a derivative thereof. Subsequently, the remainder of the second building block is removed
10 by increasing the stringency and a third building block is added under conditions allowing selective hybridisation of the third building block to the template. The fourth step results in a transfer of the functional entity R_3 to the previous transferred R_2 .

15 A library may be generated as outlined in the embodiment according to fig. 1.

In a preferred embodiment of the present invention, the template is segregated into two regions, as illustrated in fig. 6. Each of the two regions comprises two or more codons in sequence. A plurality of building blocks is pro-
20 vided. Each of the building blocks comprises an anti-codon capable of recognising a codon of the template. The building blocks may also be provided with a tail of non-specific base-pairing nucleobases and/or non-specific base-pairing nucleobases may be provided in the linker. The non-specific nucleobases are preferably provided so as to ensure that each of the building
25 blocks has essentially the same annealing temperature. When the anti-codon is distanced from the reactive group, the linker is preferably provided with non-specific base-pairing nucleobases. The non-specific base-pairing nucleobases ensures that the reactive group will be restricted in moving in the space and serves as the second pair of the molecular affinity pair. The
30 presence of the non-specific nucleobases also enables the generation of a library.

In a first step, a building block (OA1) provided with a reactive group is annealed to the template. The codon of the template is specifically recognized by the anti-codon of the building block and conditions are provided which ensures that a hybridisation will occur. Also, the part of the linker or tail comprising non-specific pairing nucleobases, such as inosine, anneals to the template. At a terminal point of the building block the reactive group is provided. Preferably, the reactive group is part of a functional entity. Still more preferred, the reactive group of the functional entity is attached to the linker of building block.

10

A second building block (OB1) is annealed to the second coding region of the template. The incorporation of the second building block may be performed prior to, simultaneously with or subsequent to the annealing of the first building block to the template. The functional entity of the second building block is provided at a terminal point of the linker, however, with a polarisation opposite to the first building block. E.g. if the reactive group of the first building blocks is attached to the 5' end, then the functional entity of the second building block is attached to the 3' end. The template and the two building blocks are designed such that the reactive groups come into close proximity. Preferably, the nucleotides to which the reactive groups is attached is spaced no more than 2 nucleotides, more preferred a single nucleotide and most preferred no nucleotide. When there is no space between the oligonucleotides, i.e. the 5' end of the first building block abuts the 3' end of the second building block, the reactive groups are provide close to each other. The close proximity will provide for a high local concentration which will increase the probability for a reaction to occur. Moreover, the close proximity will reduce the probability of side reaction to occur.

15

20

25

30

The assembly of the template annealed to the building blocks is subjected to conditions that will provide for a connection between the first building block and the functional entity of the second building block by a reaction involving the reactive group of the first building block and the reactive group of second

building block functional entity. The connection may result as a direct reaction between the reactive groups or the connection may be obtained through a suitable "fill-in" molecular entity. Following the creation of the connection, a linkage is cleaved to obtain a nascent templated molecule. The cleavage is conducted such that the nascent templated molecule is situated on the second building block (OB1). The connection and cleavage step is preferably conducted simultaneously. When it is intended to prepare a polymer, the reactive group of the first building block is preferably part of a functional entity and the reactive group is preferably connected to the linker. Moreover the functional entity of the first building block is preferably a leaving group of the reaction, resulting in a transfer of functionality from the first building block to the second building block. In other words, the functional entity of the first building block will be a precursor for the functionality eventually appearing in the nascent template-directed molecule.

After the transfer of functionality, the remaining parts of the first building block are removed. Preferably, the first building block is removed selectively, i.e. the second building block remains hybridised to the template while the first building block is separated from the template. The selective removal of the remaining parts of the building block may be performed in a number of ways. As an example the anti-codon and optionally the linker and tail may be prepared partly or totally of RNA, while the template is prepared of DNA. The RNA strand of the DNA:RNA hybrid may be cleaved at one or more sites by a RNase, such as RNase H, RNase A, RNase1, weak alkaline conditions (pH 9-10 at 30°C in 1 hr), or aqueous $\text{Pb}(\text{Ac})_2$ (2mM, room temperature, 30 min). The other strand, that is, the anti-codon, the linker and the tail of the second building block may be constructed of other nucleotides than RNA. An example is deoxyribonucleic acid (DNA) having all or a part of the natural phosphodiester internucleoside linkages substituted with a phosphorothioate (-O-P(=S)(OH)-O-) linkage. The sulphur substituted linkage may be cleaved by a suitable agent, such as aqueous iodine or iodoethanol. An alternative

example is to use a methylphosphonate in the internucleoside linker and cleave with piperidine.

5 After the removal of the remaining elements of the first building block, a third building block (OA2) may be annealed to the coding region previously harbouring the first building block. The third building block comprises an anti-codon which specifically binds to a codon different from the codon which annealed to the anti-codon of the first building block. The third building block will usually have a linker comprising a section with non-specific base-pairing nucleobases and the oligonucleotide will preferably be degradable in the same manner as the first building block. The hybridisation of the third anti-codon to the template provides the reactive group in close proximity with a reactive group of the nascent template-directed molecule harboured by the second building block. A connection is subsequently established by a reaction involving the two reactive groups followed by a cleavage of a linkage. The establishing of the linkage and the cleavage is preferably conducted in a single step resulting in a transfer of the nascent templated molecule to the functional entity of the third building block.

10

15

20 After the nascent templated molecule has been transferred to the third building block the remaining parts of the second building block is selectively removed and the system is ready for a fourth round. The method may be repeated a sufficient number of times to obtain the desired length and properties of the templated molecule. In a final step the last oligonucleotide comprising the anti-codon may be coupled covalently to the template. The coupling may be provided by a SS bridge.

25

Fig. 7 illustrates the general embodiment according to fig. 6. As an example the production of a β -peptide is chosen. Initially, a template strand of DNA is provided. Also provided is first and second building block. The first building block comprises a RNA section comprising the anti-codon and stretches of tailing universal bases. The functional entity of the first building block is a β -

30

amino acid precursor. The amino group is protected and the acid group is connected in an ester bond to an alcohol group of a phenolic group. The second building block comprises a DNA section having one or more internucleoside linkages which comprises a thiophosphate group. The second building block also comprises an amino acid precursor. The amino group is unprotected and available for reaction.

In a first step the template and the two building blocks are annealed together. Subsequently, conditions are provided which facilitate a reaction between the amine group of the second building block and the ester bond of the first building block. The reaction results in a simultaneous connection and cleavage. i.e. the precursor for the β -amino acid of the first building block is transferred to the amine group of the second building block and a peptide bond is formed.

In a third step the assembly is treated with RNase, which will degrade the RNA strand comprising the first codon. Subsequently, the RNase is removed from the media together with the oligonucleotide debris and a third building block (A2) is added. Suitably, the RNase can be immobilized on a solid support, because the removal of the RNase for the media easily can be performed, e.g. by simply separating the beads with immobilized RNase by filtration.

In a fourth step, conditions are provided by which the third building block anneals to the template. The third building block comprises an RNA strand. The DNA strand contains the anticodon which anneals to codon A2 and stretches of universal bases. The functional entity of the third building block is of the same type as used in the second building block, i.e. a precursor for β -amino acid having the amine unprotected and the acid group connected in an ester bonding to a phenolic OH group. The free amine group is available for reaction and selecting appropriate conditions will provide for a reaction of the amine group with the ester group, as shown by the arrow.

The fifth step involves a direct reaction between the amine group and the ester to obtain a transfer of the protected dipeptide to the amine group of the third building block, whereby a tripeptide is created.

5

A sixth step involves degradation of the remainder of the second building block by treatment with aqueous iodine. After removal of debris and excess iodine from the media the cycle may be repeated as appropriate until a β -peptide of a desired length is obtained. In a final step, the building block harbouring the formed β -peptide can be covalently attached to the template, e.g. by the providing the template as well as the final building block with SH groups, which may be coupled to form a -S-S- bridge.

10

A library can be formed based on the technique shown in Fig. 7 and a selection can be performed.

15

Fig. 8 shows different classes of reactions which mediate transfer of a functional entity from a building block to another entity, or to an anchorage point. For simplicity reasons only, the receiving entity is shown as a building block; it is to be understood that the receiving entity can be covalently attached to the template as well. The reactions have been grouped into three different classes: Nucleophilic substitutions, addition-elimination reactions, and transition metal catalyzed reactions.

20

(A) Reaction of nucleophiles with carbonyls. As a result of the nucleophilic substitution, the functional group R is translocated to the monomer building block initially carrying the nucleophile.

25

(B) Nucleophilic attack by the amine on the thioester leads to formation of an amide bond, in effect translocating the functional group R of the thioester to the other monomer building block.

30

(C) Reaction between hydrazine and β -ketoester leads to formation of pyrazolone, in effect translocating the R and R' functional groups to the other monomer building block.

- (D) Reaction of hydroxylamine with β -ketoester leads to formation of the isoxazolone, thereby translocating the R and R' groups to the other monomer building block.
- 5 (E) Reaction of thiourea with β -ketoester leads to formation of the pyrimidine, thereby translocating the R and R' groups to the other monomer building block.
- (F) Reaction of urea with malonate leads to formation of pyrimidine, thereby translocating the R group to the other monomer building block.
- 10 (G) Depending on whether $Z = O$ or $Z = NH$, a Heck reaction followed by a nucleophilic substitution leads to formation of coumarin or quinolinon, thereby translocating the R and R' groups to the other monomer building block.
- 15 (H) Reaction of hydrazine and phthalimides leads to formation of phthalhydrazide, thereby translocating the R and R' groups to the other monomer building block.
- (I) Reaction of amino acid esters leads to formation of diketopiperazine, thereby translocating the R group to the other monomer building block.
- 20 (J) Reaction of urea with α -substituted esters leads to formation of hydantoin, and translocation of the R and R' groups to the other monomer building block.
- 25 (K) Alkylation may be achieved by reaction of various nucleophiles with sulfonates. This translocates the functional groups R and R' to the other monomer building block.
- (L) Reaction of a di-activated alkene containing an electron withdrawing and a leaving group, whereby the alkene is translocated to the nucleophile.
- 30 (M) Reaction of disulfide with mercaptane leads to formation of a disulfide, thereby translocating the R' group to the other monomer building block.

- (N) Reaction of amino acid esters and amino ketones leads to formation of benzodiazepinone, thereby translocating the R group to the other monomer building block.
- 5 (O) Reaction of phosphonates with aldehydes or ketones leads to formation of substituted alkenes, thereby translocating the R" group to the other monomer building block.
- (P) Reaction of boronates with aryls or heteroaryls results in transfer of an aryl group to the other monomer building block (to form a biaryl).
- 10 (Q) Reaction arylsulfonates with boronates leads to transfer of the aryl group.
- (R) Reaction of boronates with vinyls (or alkynes) results in transfer of an aryl group to the other monomer building block to form a vinylarene (or alkynylarene).
- 15 (S) Reaction between aliphatic boronates and arylhalides, whereby the alkyl group is translocated to yield an alkylarene.
- (T) Transition metal catalysed alpha-alkylation through reaction between an enolether and an arylhalide, thereby translocating the aliphatic part.
- 20 (U) Condensations between e.g. enamines or enolethers with aldehydes leading to formation of alpha-hydroxy carbonyls or alpha,beta-unsaturated carbonyls. The reaction translocates the nucleophilic part.
- (V) Alkylation of alkylhalides by e.g. enamines or enolethers. The reaction translocates the nucleophilic part.
- 25 (W) [2+4] cycloadditions, translocating the diene-part.
- (X) [2+4] cycloadditions, translocating the ene-part.
- (Y) [3+2] cycloadditions between azides and alkenes, leading to triazoles by translocation of the ene-part.
- 30 (Z) [3+2] cycloadditions between nitriloxides and alkenes, leading to isoxazoles by translocation of the ene-part.

Fig. 9 shows examples of pairs of reactive groups X and Y and the resulting bond. The schemes can be used in guiding the skilled person in the selection

of appropriate reactive group for formation of connections. The formations formed are in one aspect of the invention adapted such that they are cleavable or selectively cleavable. In another aspect, the connections are adapted to be durable. The first aspect applies in the embodiments shown in Figs. 1 to 5, whereas the first as well as the second aspect may apply for the embodiments shown in Figs. 6 and 7.

Fig. 10 shows examples of cleavable connections and the conditions for their cleavage and the resulting products.

Fig. 11 shows examples of different methods for loading a derivatised oligonucleotide with a functional entity. Oligonucleotides with the reactive groups indicated to which a functional entity can be added according to the schemes are commercially available.

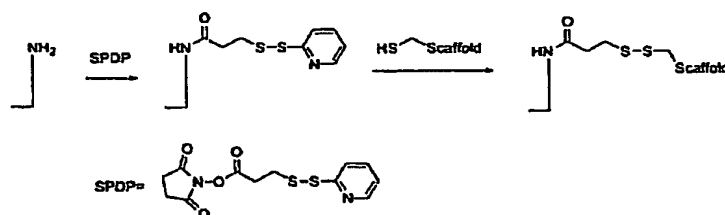
Fig. 12 shows a typical panning protocol for selection of templated molecules. The details of Fig. 12 are disclosed above.

Example 1

Synthesis of a scaffolded molecule comprising 3 DNA-encoded functional entities.

This example describes the templated synthesis of a scaffolded molecule comprising 3 DNA encoded substituents using the set-up described in figure 1. A hexameric scaffold peptide with the sequence, CysPhePheLysLysLys, was synthesised by standard solid-phase Fmoc peptide chemistry. The scaffold peptide comprises a -SH group on the cysteine side chain, said -SH group being used for coupling the scaffold peptide to an amine-bearing oligonucleotide serving as anticodon and linker. Each of the three lysine moieties comprises an amino group in the side chain. The amine groups are used as reactive groups for the formation of a connection to functional entities emanating from building blocks.

The N- and C-terminus of the peptide was initially capped to avoid any participation in the reactions to follow and subsequently purified by reverse phase-HPLC. The scaffold peptide was covalently attached to DNA oligonucleotide using the scheme shown schematically below. For illustrative purposes, the scaffold is indicated as HS-Scaffold.



10

5 nmol of oligodeoxynucleotide O1, YCGATGGATGCTCCAGGTCGC where Y = C6 amino-group (Glen Research, cat. # 10-1039-90) in 100 mM Hepes-OH pH 7.5 is incubated with 20 mM Succinimidyl-propyl-2-dithiopyridyl (SPDP, Molecular probes) dissolved in DMSO for 3 hours at 25 °C. Excess SPDP is removed by triple extraction using 5 volumes of ethylacetate. The sample is further purified using a Bio-rad Microspin 6 column equilibrated in H₂O.

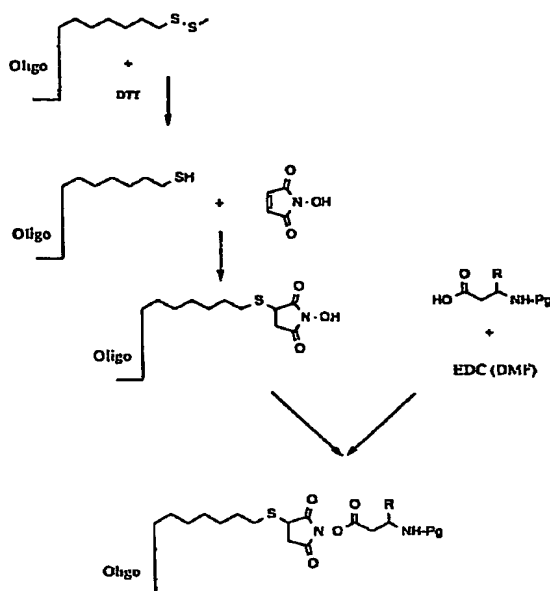
20

The oligonucleotide-scaffold conjugate is synthesised by incubating 1 µmol hexapeptide with 5 nmol SPDP activated oligonucleotide in 100 mM Hepes-OH pH 7.5 for 2 hours at 25 °C. Excess peptide is removed by double sodium-acetate/ethanol precipitation of the scaffold-DNA complex according to standard procedure. The loading was verified by Electrospray Mass Spectrometry (ES-MS).

25

Calculated mass of oligo-scaffold complex: 7240.27 Da
 Observed mass of oligo-scaffold complex: 7238.85 Da
 Estimated loading efficiency: 92 %

Three building block oligonucleotides O2, O3 and O4 were loaded with three different building blocks using the scheme shown below.



5

The three building block oligonucleotides O2, BGAGCATCCATCGX, O3, BCTGGAGCATCCATCGX and O4, BGCGACCTGGAGCATCCATCGX each comprising a 5' biotin group (B) and a 3' end C6 S-S thiol modifier (X)(Glen Research# 10-1936-90) was provided. Initially, a free thiol-group was formed in a reduction-step by incubating 10 nmol of each oligonucleotide with 100 mM DTT in a 100 mM sodium-phosphate buffer pH 8.0 at 37 °C for 30 min. Subsequently, buffer and DTT are removed using Bio-rad Microspin 6 columns. Next, the free thiol-group was reacted with the Michael-acceptor of N-hydroxymaleimide to form the N-hydroxysuccinimid-oligo complex using the following procedure: The thiol-oligo is incubated in 100 mM Hepes-OH pH 7.5 and 100 mM N-hydroxymaleimide (Merck) at 25 °C for 2 hours. Subsequently, buffer and excess NHM are removed using Bio-rad Microspin 6 columns. Synthesis of oligo-NHS complexes are verified by ES-MS.

Calculated mass of O2-NHS: 4243.47 Da

Observed mass of O2-NHS: 4343.88 Da

Estimated loading efficiency: > 95 %

5

Calculated mass of O3-NHS: 5265.06 Da

Observed mass of O3-NHS: 5265.32 Da

Estimated loading efficiency: > 95 %

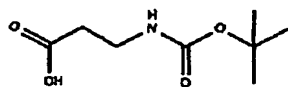
10

Calculated mass of O4-NHS: 6816.05 Da

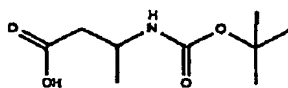
Observed mass of O4-NHS: 6816.46 Da

Estimated loading efficiency: > 95 %

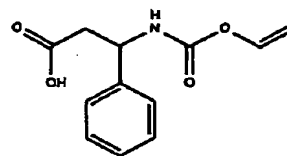
The NHS-oligonucleotides O2 to O4 was loaded with functional entities BB1 to BB3, respectively, to forming the building blocks, using the following procedure: 100 mM functional entity is incubated with equimolar 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide-hydrochloride (EDC) in dimethylformamide (DMF) at 25 °C for 30 minutes. Subsequently, 10 µl of the mixture is incubated with 500 pmol of NHS-oligo in 100 mM NH₄-acetate buffer pH 6 at 25 °C for 10 minutes in a volume of 20 µl. Finally, excess functional entity and EDC is removed by addition of 30 µl of 100 mM sodium-phosphate pH 6 and purification using Bio-rad Microspin 6 columns equilibrated in 100 mM NH₄-acetate pH 6.



BB1



BB2



BB3

25

200 pmol of the formed building block is used immediately and added to 150 pmol of scaffold-oligo conjugate in 100 mM NH_4 -acetate, pH 6 and incubated overnight at 15°C.

- 5 After the first transfer, the reacted O2 building block oligonucleotide is removed by incubating the O1/O2 oligo complex with 50 μl pre-washed (50 mM NH_4 acetate, pH 6) Streptavidin-sepharose beads (AmershamPharmacia) at 4 °C for 10 min with gentle agitation. The sample is spun briefly and the supernatant is removed before addition of 100 μl ice-cold H_2O followed by gentle agitation for 2 min at 4 °C. The sample is again spun briefly and the supernatant is removed before addition of 100 μl H_2O followed by incubation at 80 °C for 5 min. The sample is spun briefly and the supernatant containing the scaffold oligonucleotide is transferred to a new tube and the sample is lyophilized. This procedure is iterated using O3 and O4 loaded with BB2 and BB3, respectively, except that the annealing of the oligo O3-BB2/O1-scaffold and O4-BB3/O1-scaffold is conducted at 20 °C and 30 °C, respectively.
- 10
- 15 Specific substitution of the tri-amino scaffold with the three encoded reactants is verified by ES-MS.

Claims

1. A method for synthesising a bifunctional complex comprising a template-directed molecule and a template for the synthesis thereof, the method comprising the steps of:

- 5 a) providing a template comprising two or more codons in sequence, a first part of a molecular affinity pair, and a reactive group,
- b) providing two or more building blocks, each building block comprises
- i) an anti-codon capable of recognising a codon of the template,
- 10 ii) a functional entity comprising at least one reactive group,
- iii) a linker connecting the anti-codon and the functional entity,
- wherein building blocks having anti-codons intended to interact with codons of the template distal to the reactive group comprise as a section of the linker a second part of the molecular affinity pair,
- 15 c) contacting the template with a building block under conditions which allow specific hybridisation of the anti-codon of the building block to the codon of the template, and under conditions ensuring assembling of the parts of the molecule pair, if present,
- 20 d) obtaining a connection between the functional entity of the building block and the template by a reaction involving the template reactive group and the functional entity reactive group,
- e) cleaving a linkage to obtaining a nascent templated molecule,
- f) separating the parts of the molecular affinity pair,
- 25 g) repeating, for a building block having an anti-codon capable of hybridising to a new codon, steps c) to f) one or more times,
2. The method according to claim 1, wherein the first part of the affinity pair is capable of reversible interaction with the second part of the affinity pair.
- 30 3. The method according to claim 1 or 2, wherein the first part of the affinity pair is a sequence of nucleotides and the second part of the affinity pair is

a sequence of nucleotides capable of hybridising to the first part of the affinity pair.

4. The method according to claim 1 or 2, wherein the one or more at the nucleotides of the second part of the affinity pair comprise a non-specific
5 base-pairing nucleobase.
5. The method according to claim 4, wherein the non-specific pairing nucleobase is selected from the group comprising inosine, pyrene, 3-nitropyrrole, N⁶-8aza-7deazaadenine and 5-nitroindole.
6. The method according to claim 1, wherein the first part of the molecular
10 affinity pair is comprised by the two or more of the codons in sequence.
7. The method according to claim 1, wherein the codon is a sequence of nucleotides.
8. The method according to claim 1, wherein the nucleotide sequence of the first part of the molecular affinity pair comprise at least a part of the nucleotide sequence of the codon proximal to the reactive group of the template.
15
9. The method according to claim 1, wherein the reactive group of the template is covalently attached to the template.
10. The method according to claim 1, wherein the reactive group of the
20 template is a part of a functional entity or a nascent templated molecule.
11. The method according to claim 1, wherein the reactive group of the template is a part of a scaffold.
12. The method according to claim 1, wherein the reactive group of the template is non-covalently attached to the template.
- 25 13. The method according to claim 12, wherein the reactive group of the template is attached to a sequence of nucleotides, which complements a sequence of nucleotides of the template.
14. The method according to claim 13, wherein the reactive group of the template is attached to an anti-codon complementing a further codon of the
30 template.
15. The method according to claim 1, wherein the template comprises two regions of codons, the two or more codons in sequence of step a) belonging

to the first region and the second region of codons comprising one or more codon(s) for initial attachment of the reactive group of the template.

16. The method according to claim 1, wherein neighbouring codons of the codons in sequence are separated by a spacer group.

5 17. The method according to claim 16, wherein the spacer group identifies the position of a codon.

18. The method according to claim 16, wherein the spacer group comprises a sequence of one or more guanine nucleobase(s).

10 19. The method according to claim 1, wherein the number of codons of the template is 3 to 100.

20. The method according to any of the claims 1 to 19, wherein each codon is a sequence of 3 to 100 nucleotides.

21. The method according to claim 20, wherein the each codon comprises a sequence 3 to 30 nucleotides.

15 22. The method according to claim 1, wherein the second part of the molecule affinity pair in the linker of the building block is arranged proximal to the functional entity.

20 23. The method according to claim 22, wherein the second part of the molecular affinity pair is spaced from the functional entity by 0 to two nucleotides.

24. The method according to claim 1, wherein the reactive group of a building block functional entity is capable of forming a direct connection to a reactive group of the template.

25 25. The method according to claim 1, wherein the reactive group of a building block is capable of forming a connection to a reactive group of the template through a bridging fill-in group.

26. The method according to claim 1, wherein the connection and the subsequent cleavage of the linkage according to steps d) and e) results in a transfer of the functional entity to the nascent template-directed molecule.

30 27. The method according to claim 1, wherein the connection and the subsequent cleavage of the linkage according to steps d) and e) results in a

transfer of the nascent template-directed molecule to the functional entity of the building block.

28. The method according to claim 1, wherein steps d) and e) occur simultaneously.

5 29. The method according claim 1, wherein the anti-codon, the linker and the second part of the molecular affinity pair is a contiguous linear oligonucleotide.

30. The method according to claim 1, wherein the linker is attached to the functional entity via the reactive group.

10 31. The method according to claims 24 to 30, wherein the functional entity comprises a further reactive group capable of participating in a repeated formation of a connection according to steps c) to f).

32. The method according to claims 26 or 27, wherein the transfer of the functional entity of step d) involves simultaneous connecting the functional
15 entity to the template and cleavage of the junction between the functional entity and the linker.

33. The method according to any of the claims 1, wherein the contacting of the building blocks with the template involves separate addition of the individual building blocks.

20 34. The method according to any of the claim 1, wherein the contacting of the individual building blocks with the template is controlled by directing the annealing temperature.

35. The method according to claim 34, wherein the method comprise addition of all, or a substantial amount of, the building blocks to the template and
25 directing the contacting by step-wise decreasing the temperature.

36. The method according to claim 1, wherein the anti-codon of a reacted building block not harbouring the nascent template-directed molecule is removed from the template prior to repetition of steps c) to f).

37. The method according to claim 36, wherein the anti-codon is removed
30 by melting it off the template.

38. The method according to claim 36, wherein the anti-codon is at least partly digested enzymatically.

39. The method according to claim 38, wherein the anti-codon is degraded by any one of the methods selected from the group consisting of providing a DNA template and an anti-codon comprising RNA and treating the DNA:RNA duplex with an enzyme selected from RNaseH, RNaseA, RNase 1, weak alkaline conditions (pH 9-10), or aqueous $Pb(Ac)_2$; providing a DNA template and an DNA or RNA anti-codon comprising a thiophosphate in the internucleoside linker and subsequent treating with aqueous iodine; and providing a DNA or RNA template and a DNA anti-codon comprising an uracil nucleobase, treating with uracil-glycosylase and subsequent weak acid.
40. The method according to any of the claims 1 to 39, comprising the further step of connecting the templated molecule with the template which directed the synthesis thereof via a covalent link.
41. The method according to claim 40, wherein the covalent link is selectively cleavable to provide for a release of the templated molecule.
42. The method according to claim 1, wherein the templated molecule is a polymer.
43. The method according to any of the preceding claims, comprising the further step of transferring the templated molecule to an anchorage point on the template or a sequence complementing the template, to establish an effective chemical connection.
44. The method according to claim 43, wherein the complementing sequence has a higher annealing temperature than the annealing temperature of one or more of the building blocks.
45. The method according to claim 43 or 44, wherein the sequence complementing the template is covalently connected to the template.
46. The method according to claim 45, wherein the covalent link is selectively cleavable to provide for a separation of the templated molecule from the template.
47. A library of different complexes, each complex comprising a template-directed molecule and a template for the synthesis thereof, said library being obtainable by processing a plurality of different templates and a plurality of building blocks according to any of the claims 1 to 46.

Abstract

Disclosed is a method for synthesising a bifunctional complex. The complex comprises a template as well as a molecule, the synthesis of which being directed by the template. The synthesis of the complex requires initially a) a

5 template comprising two or more codons in sequence, a first pair of a molecular affinity pair, and a reactive group; and b) two or more building blocks, each building block comprises i) an anti-codon capable of recognising a codon of the template, ii) a functional entity comprising at least one reactive group, and iii) a linker connecting the anti-codon and the functional entity,

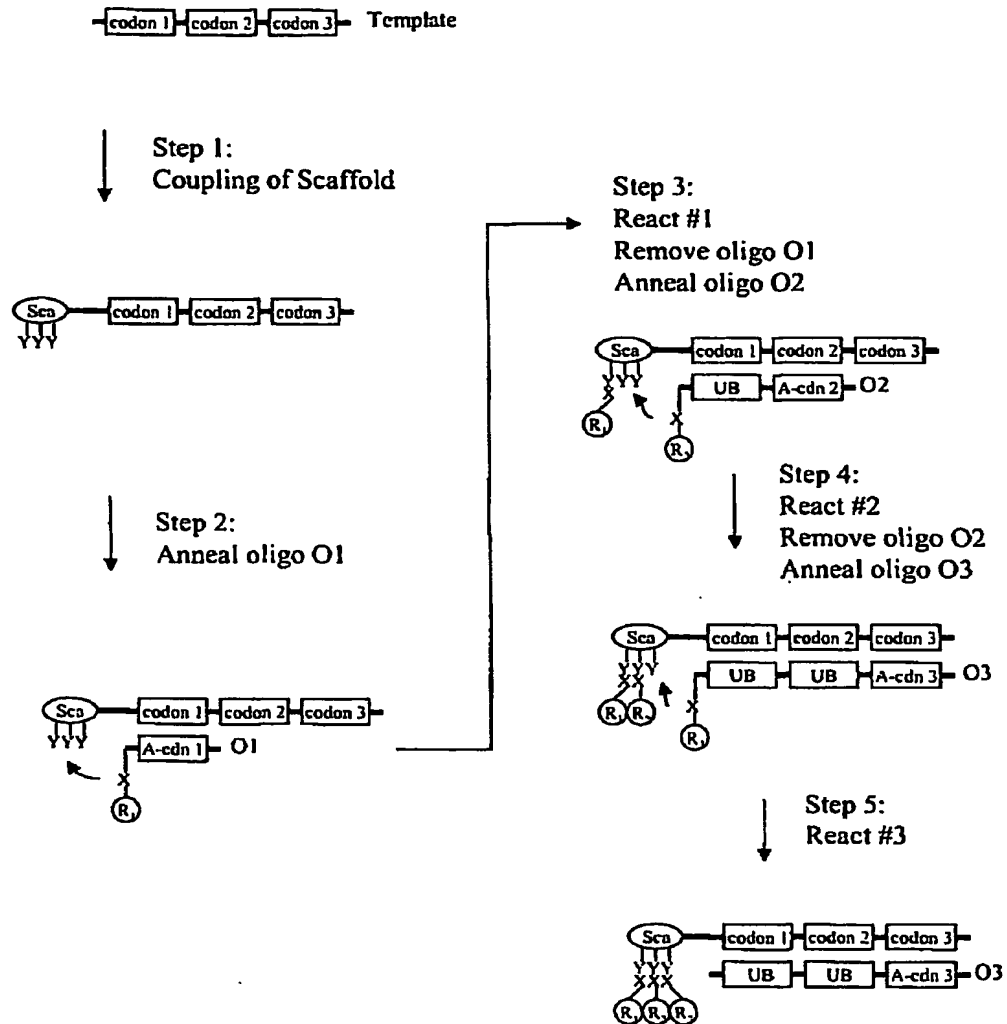
10 wherein building blocks having anti-codons intended to interact with codons of the template distal to the reactive group comprise as a section of the linker a second part of the molecular affinity pair. The synthesis proceeds by c) contacting the template with a building block under conditions which allow specific hybridisation of the anti-codon of the building block to the codon of

15 the template, and under conditions ensuring assembling of the parts of the molecule pair, if present; d) obtaining a connection between the functional entity of the building block and the template by a reaction involving the template reactive group and the functional entity reactive group, e) cleaving a linkage to obtaining a nascent templated molecule, f) separating the parts of

20 the molecular affinity pair, and g) repeating, for a building block having an anti-codon capable of hybridising to a new codon, steps c) to f) one or more times. The complexes obtainable according the invention may be used in the generation of a library which may be enriched with regard to preferred complexes using molecular evolution techniques.

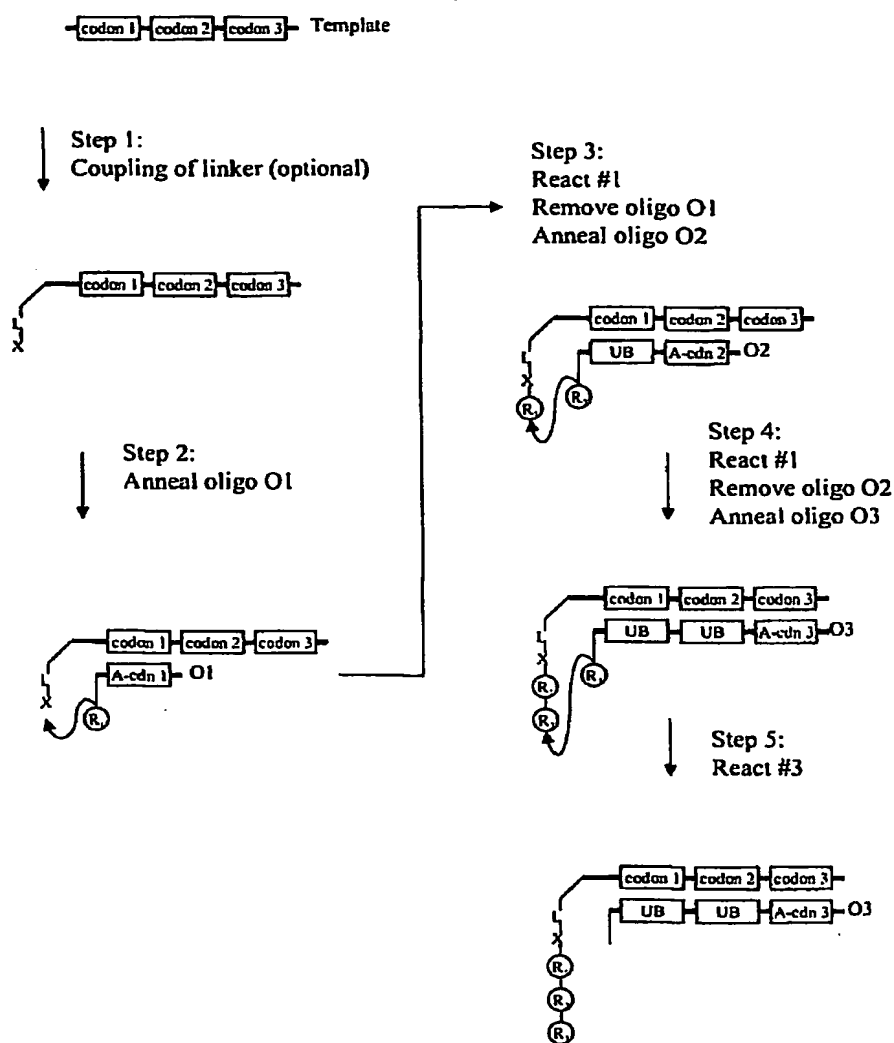
1/25

Fig.1



2/25

Fig. 2



3/25

Fig. 3

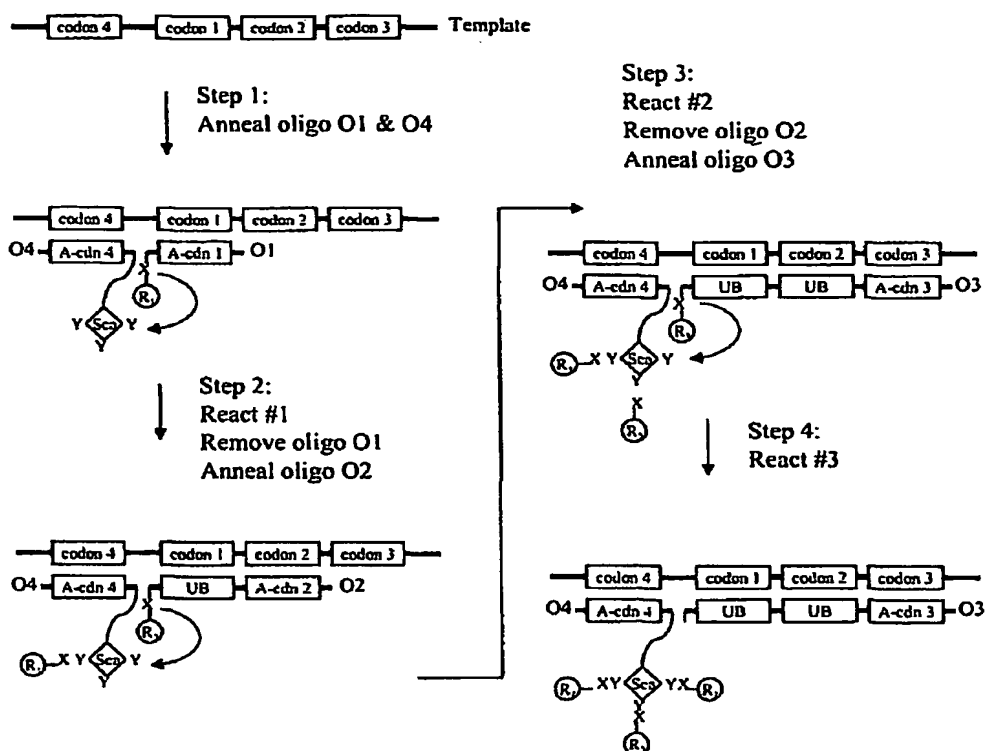
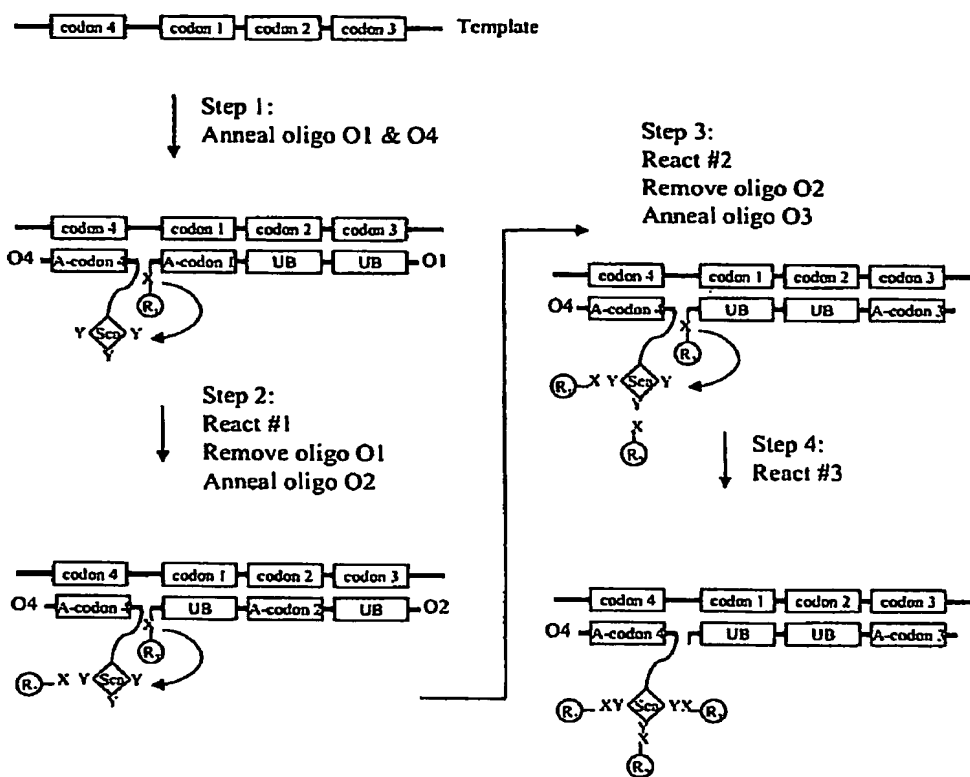


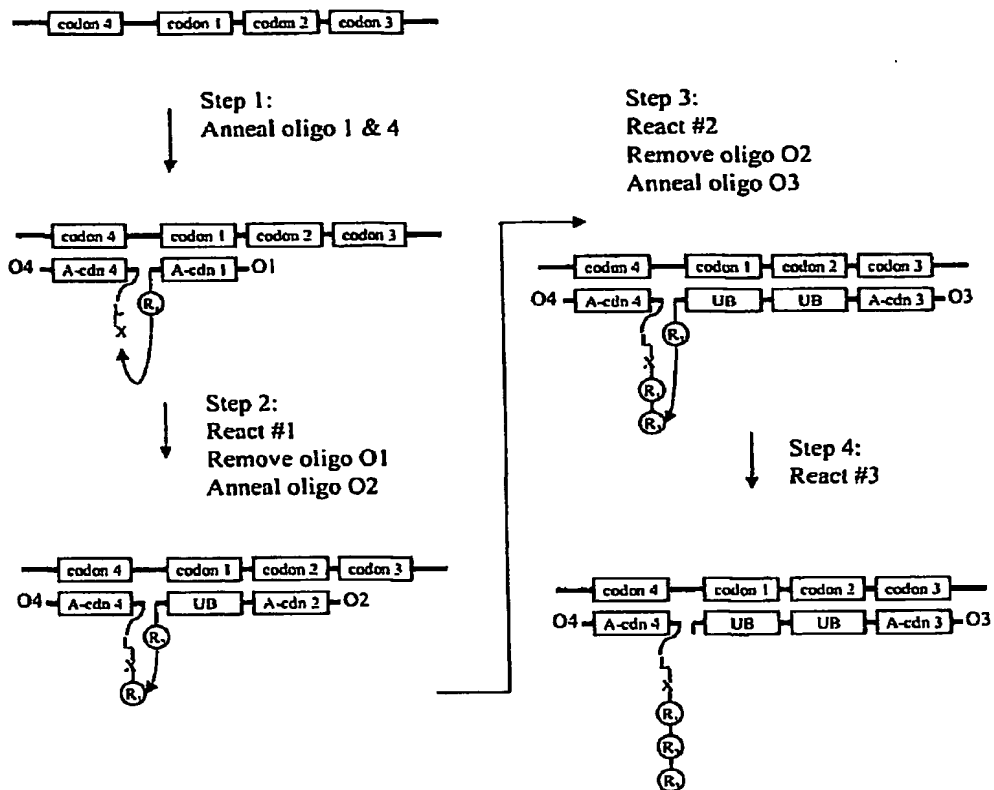
Fig 4



5/25

Modtaget
12 SEP. 2002
PVS

Fig.5



12 SEP. 2002

PVS

6/25

Fig. 6

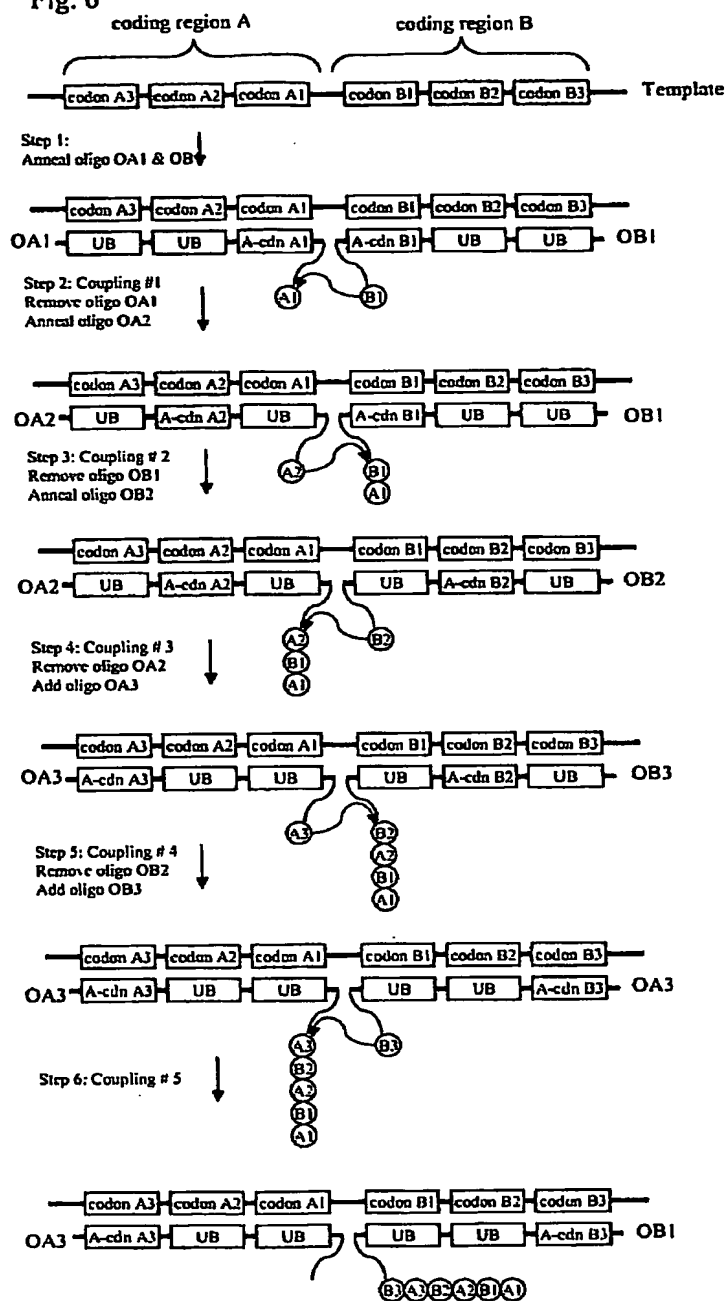


Fig. 7

7/25

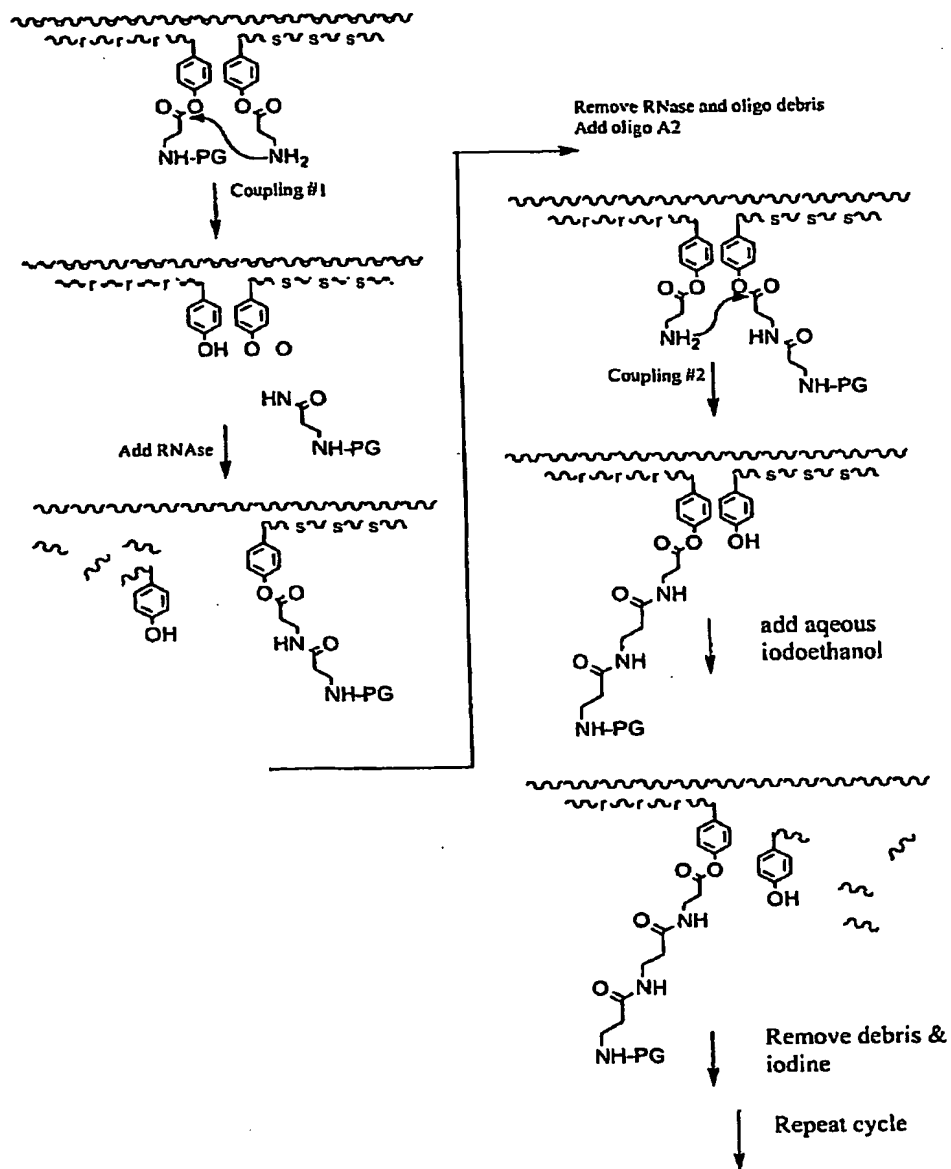
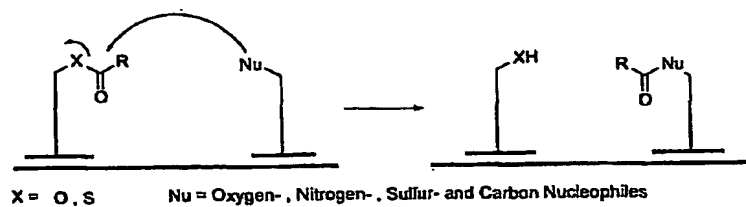
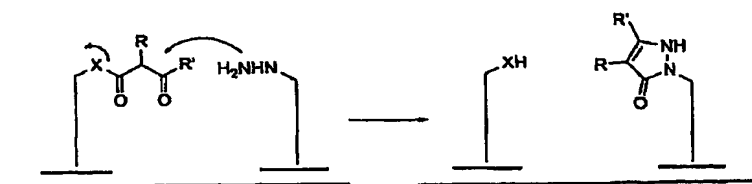


Fig. 8**A. Acylating monomer building blocks - principle****B. Acylation****Amide formation by reaction of amines with activated esters****C. Acylation****Pyrazolone formation by reaction of hydrazines with β -Ketoesters**

Modtaget

12 SEP. 2002

PVS

9/25

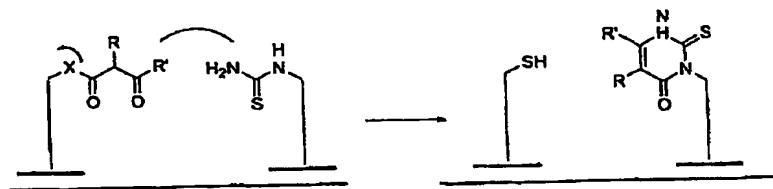
D. Acylation

Isoxazolone formation by reaction of hydroxylamines with β -Ketoesters



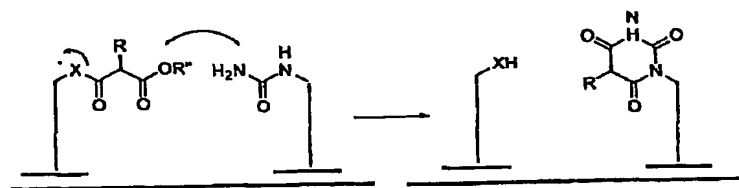
E. Acylation

Pyrimidine formation by reaction of thioureas with β -Ketoesters



F. Acylation

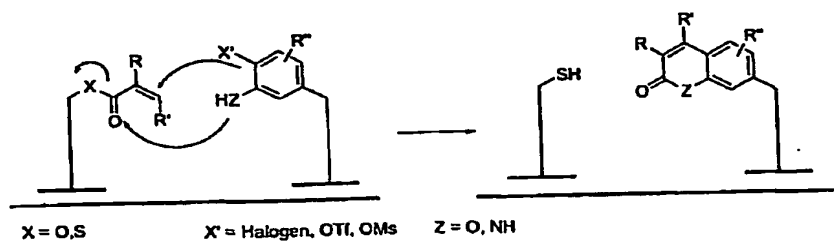
Pyrimidine formation by reaction of ureas with Malonates



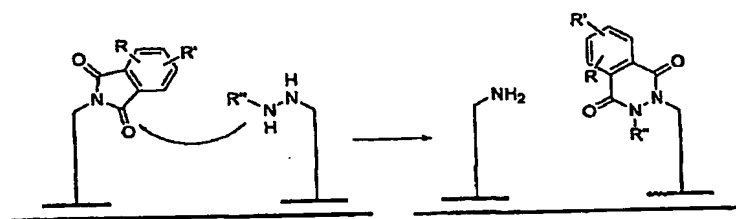
10/25

G. Acylation**PVS**

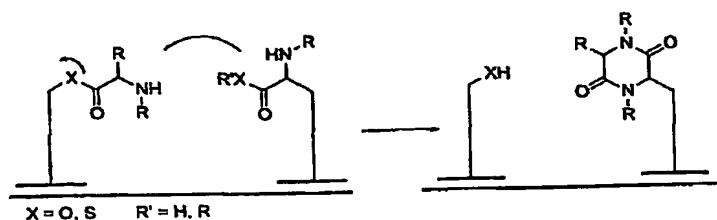
Coumarine or quinolinon formation by a Heck reaction followed by a nucleophilic substitution

**H. Acylation**

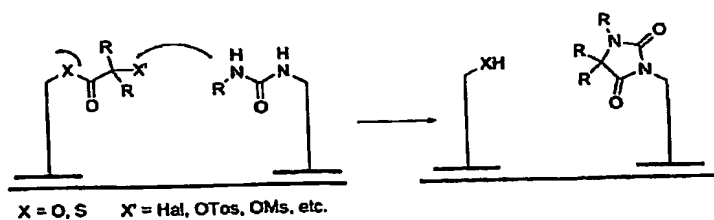
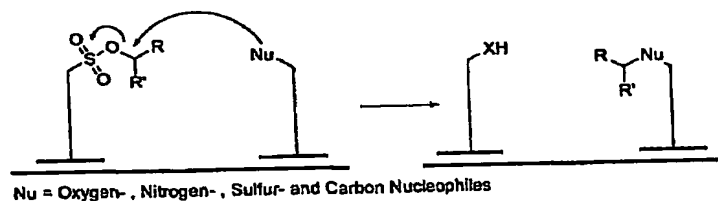
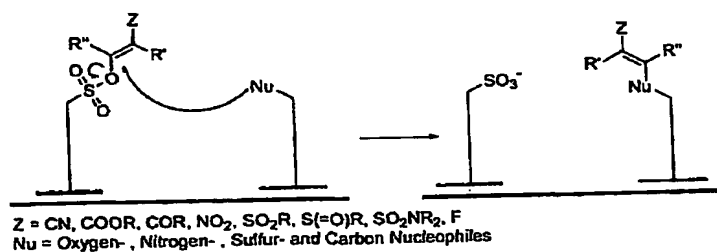
Phthalhydrazide formation by reaction of Hydrazines and Phthalimides

**I. Acylation**

Diketopiperazine formation by reaction of Amino Acid Esters



11/25

J. Acylation**Hydantoin formation by reaction of Urea and α -substituted Esters****K. Alkylating monomer building blocks - principle****Alkylated compounds by reaction of Sulfonates with Nucleophiles****L. Vinylating monomer building blocks - principle**

12/25

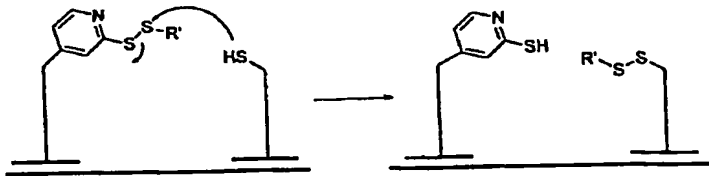
Modtaget

12 SEP. 2002

PVS

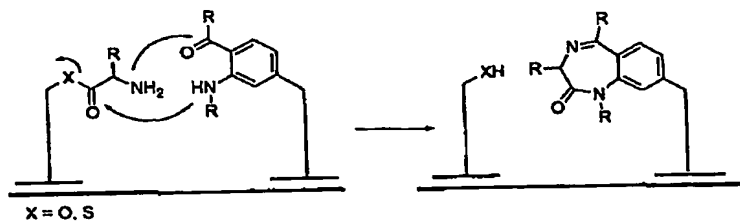
M. Heteroatom electrophiles

**Disulfide formation by reaction of Pyridyl disulfide with
Mercaptanes**



N. Acylation

**Benzodiazepinone formation by reaction of Amino Acid Esters
and Amino Ketones**



13/25

Modtaget

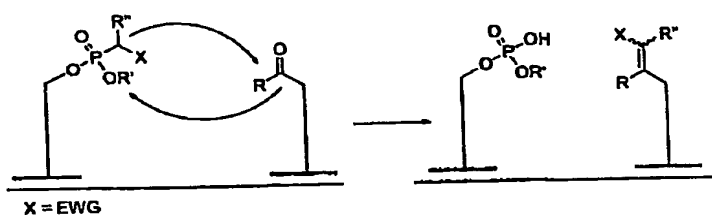
12 SEP. 2002

PVS

Addition to carbon-hetero multiple bonds

O. Wittig/Homer-Wittig-Emmons reagents

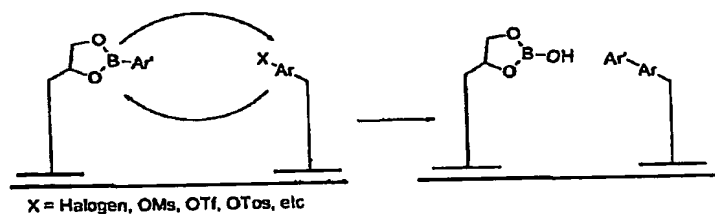
Substituted alkene formation by reaction of Phosphonates with Aldehydes or Ketones



Transition metal catalysed reactions

P. Arylation

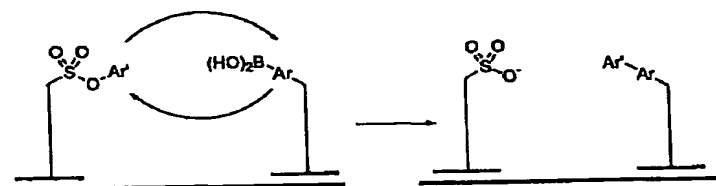
Biaryl formation by the reaction of Boronates with Aryls or Heteroaryls



14/25

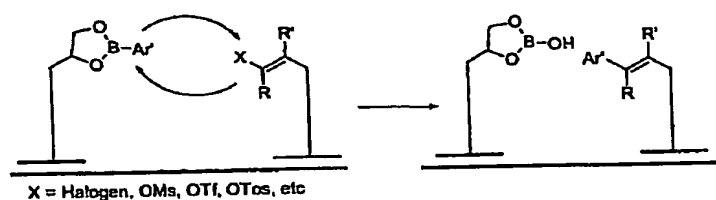
Q. Arylation

Biaryl formation by the reaction of Boronates with Aryls or Heteroaryls



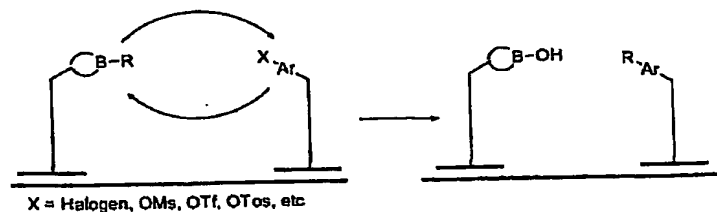
R. Arylation

Vinylarene formation by the reaction of alkenes with Aryls or Heteroaryls



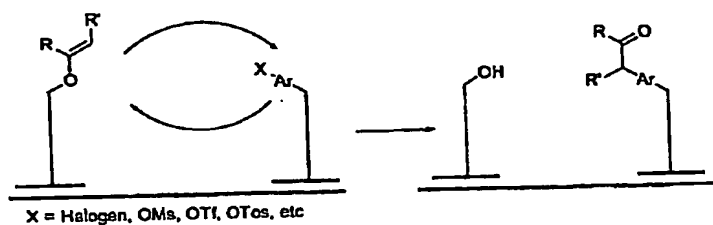
S. Alkylation

Alkylation of arenes/heterarenes by the reaction with Alkyl boronates



T. Alkylation

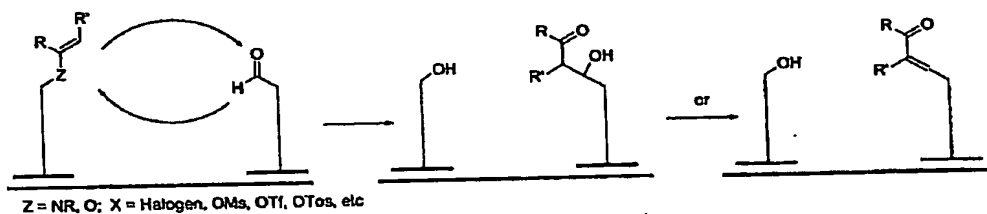
Alkylation of arenas/hetarenes by reaction with enolethers



Nucleophilic substitution using activation of nucleophiles

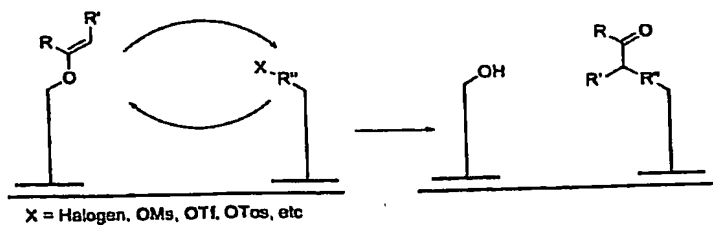
U. Condensations

Alkylation of aldehydes with enolethers or enamines

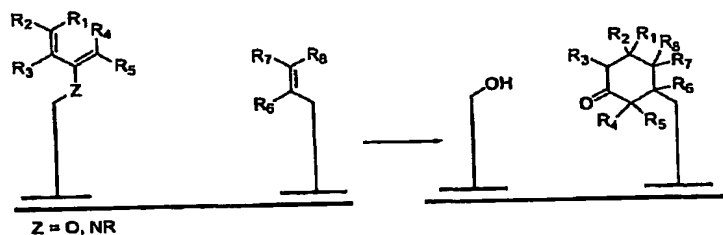
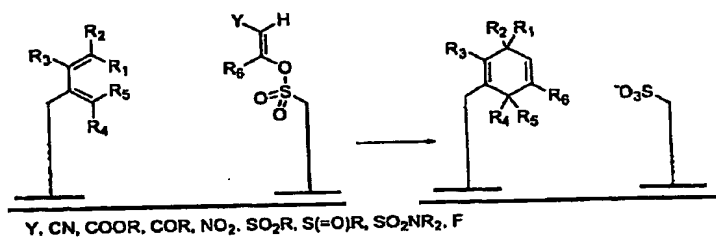
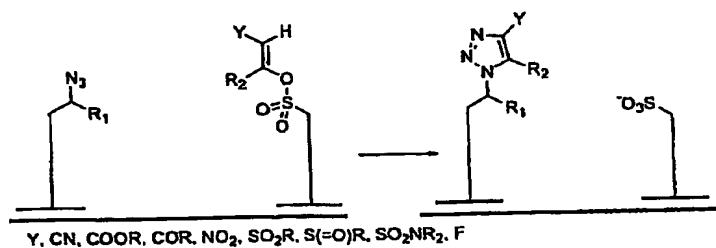


V. Alkylation

Alkylation of aliphatic halides or tosylates with enolethers or enamines



16/24

Cycloadditions**W. [2+4] Cycloadditions****X. [2+4] Cycloadditions****Y. [3+2] Cycloadditions**

17/25

Modtaget

12 SEP. 2002

Z. [3+2] Cycloadditions

PVS

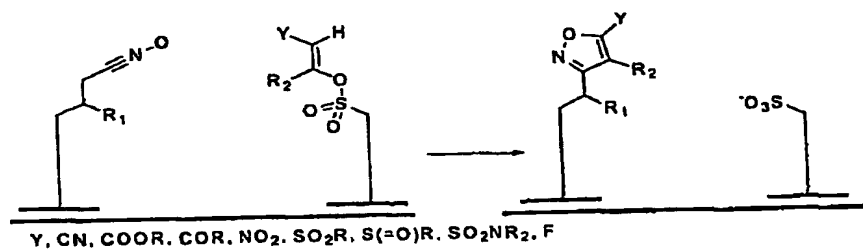
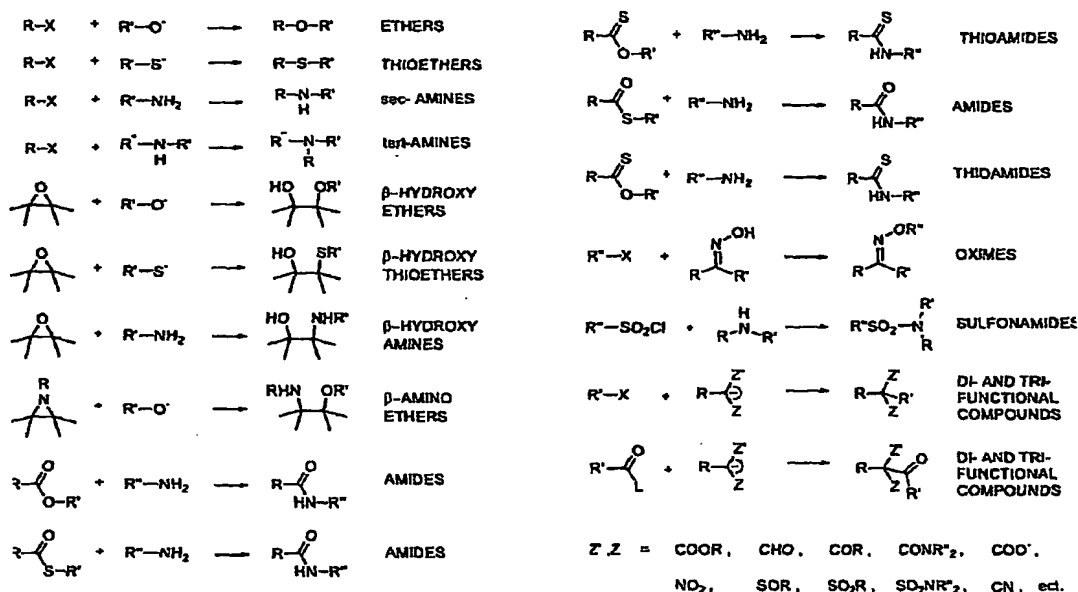


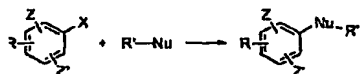
Fig. 9

Nucleophilic substitution reaction



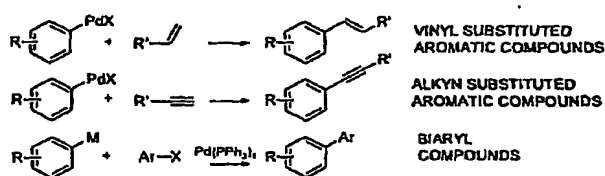
Aromatic nucleophilic substitution

SUBSTITUTED AROMATIC COMPOUNDS



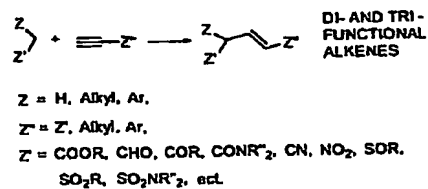
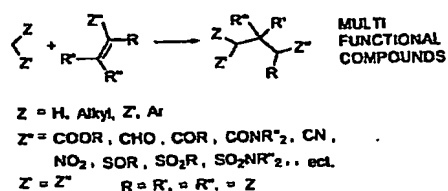
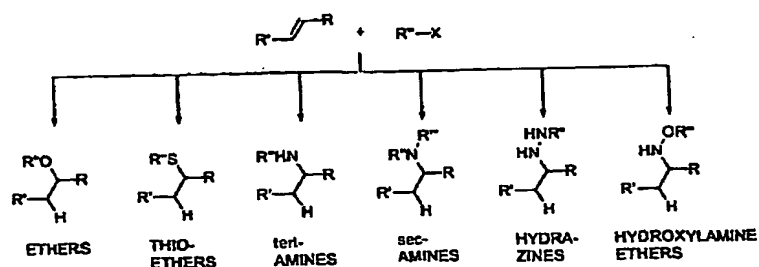
Nu = Oxygen-, Nitrogen-, Sulfur- and Carbon Nucleophiles
 X = F, Cl, Br, I, OSO₂CH₃, OSO₂CF₃, OSO₂TOL, ... etc.
 Z, Z = COOR, CHO, COR, CONR'₂, COO', CN,
 NO₂, SOR, SO₂R, SO₂NR'₂, ... etc.

Transition metal catalysed reactions

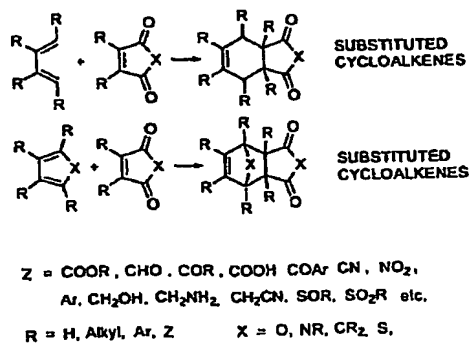
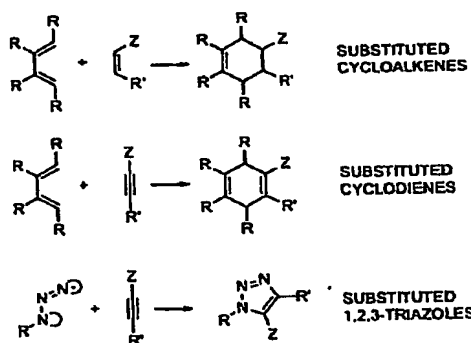


19/25

Addition to carbon-carbon multiple bonds



Cycloaddition to multiple bonds



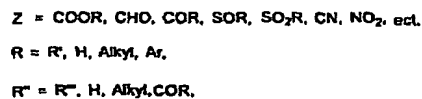
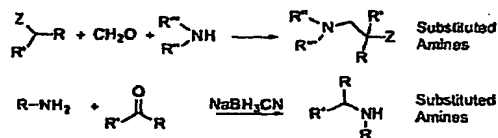
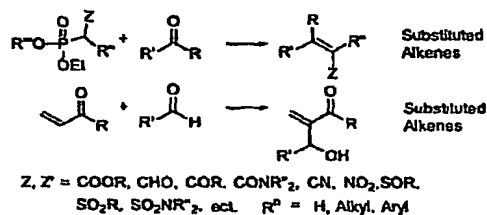
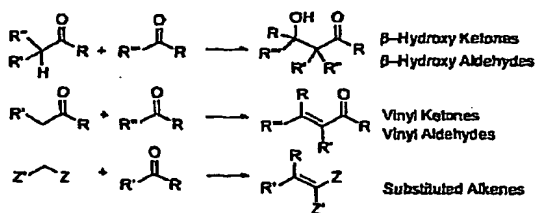
Modtaget

12 SEP. 2002

PVS

20/25

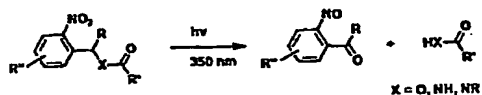
Addition to carbon-hetero multiple bonds



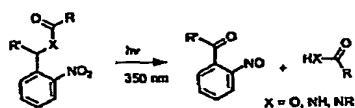
21/25

Fig. 10 Cleavable Linkers

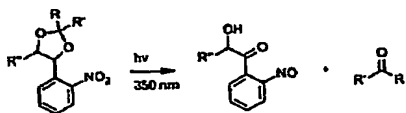
A. Linker for the formation of Ketones, Aldehydes, Amides and Acids



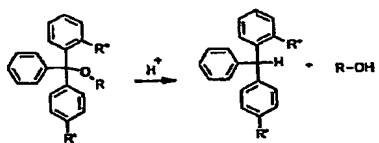
B. Linker for the formation of Ketones, Amides and Acids



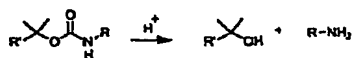
C. Linker for the formation of Aldehydes and Ketones



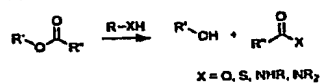
D. Linker for the formation of Alcohols and Acids



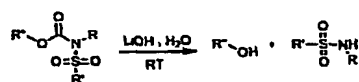
E. Linker for the formation of Amines and Alcohols



F. Linker for the formation of Esters, Thioesters, Amides, and Alcohols

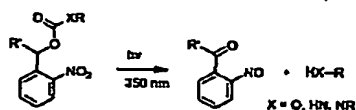


G. Linker for the formation of Sulfonamides and Alcohols

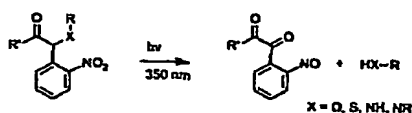


22/25

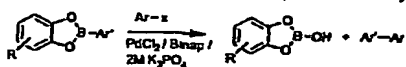
H. Linker for the formation of Ketones, Amines and Alcohols



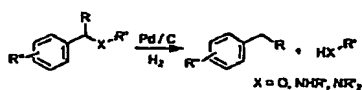
I. Linker for the formation of Ketones, Amines, Alcohols and Mercaptanes



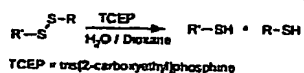
J. Linker for the formation of Biaryl and Bihetaryl



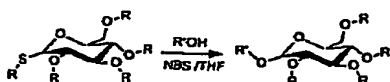
K. Linker for the formation of Benzyles, Amines, Anilins Alcohols and Phenoles



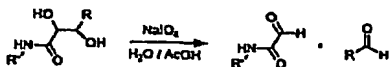
L. Linker for the formation of Mercaptanes



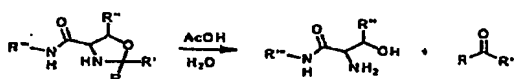
M. Linker for the formation of Glycosides



N. Linker for the formation of Aldehydes and Glyoxylamides

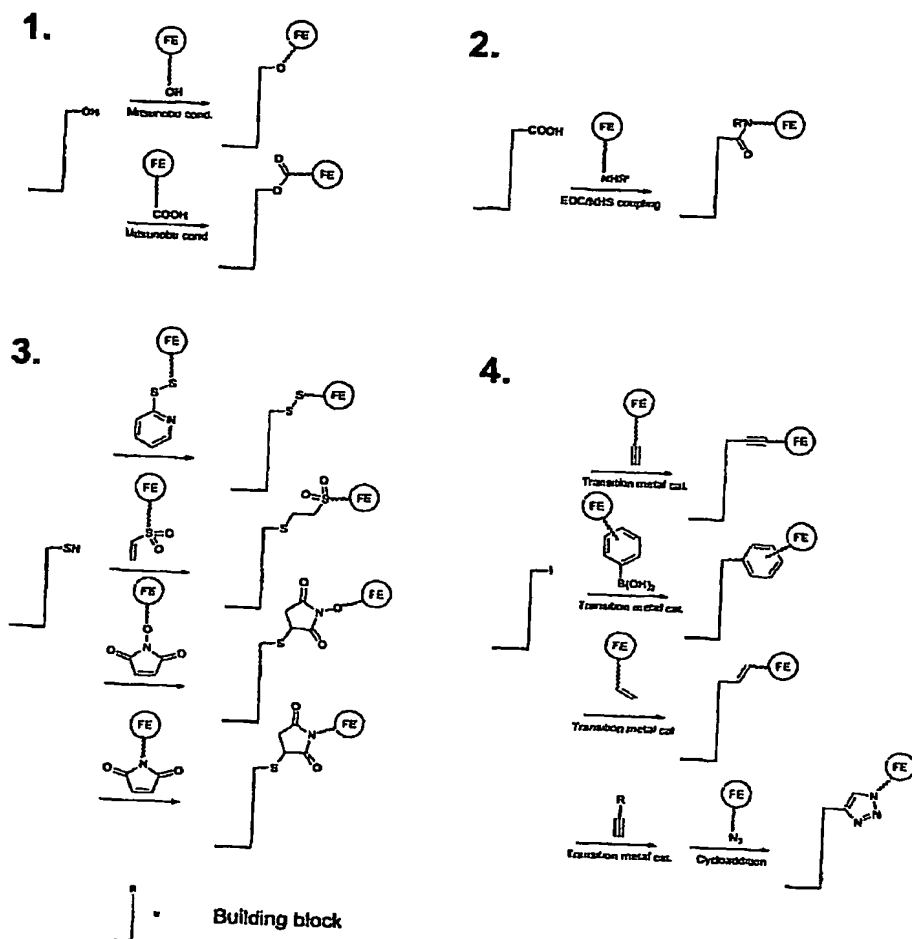


O. Linker for the formation of Aldehydes, Ketones and Aminoalcohols



23/25

Fig. 11



24/25

5.

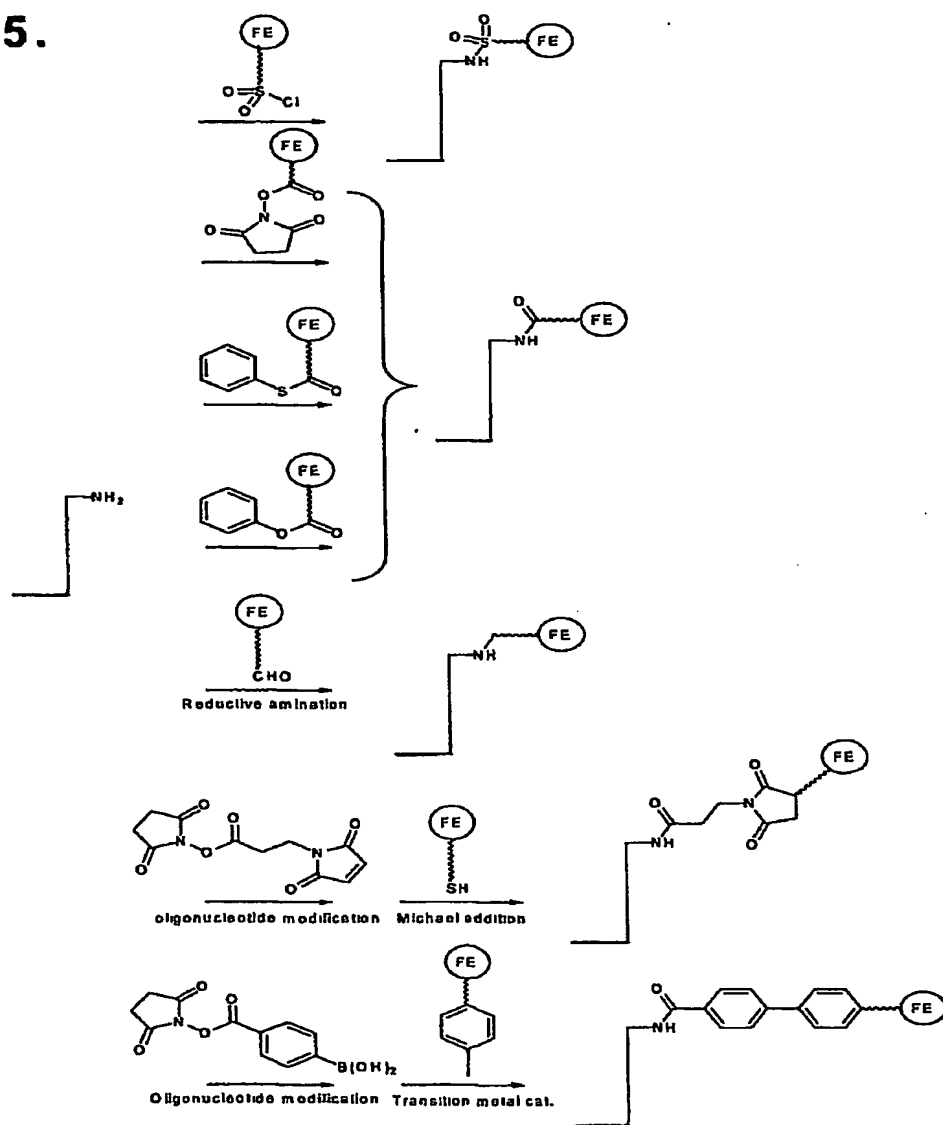
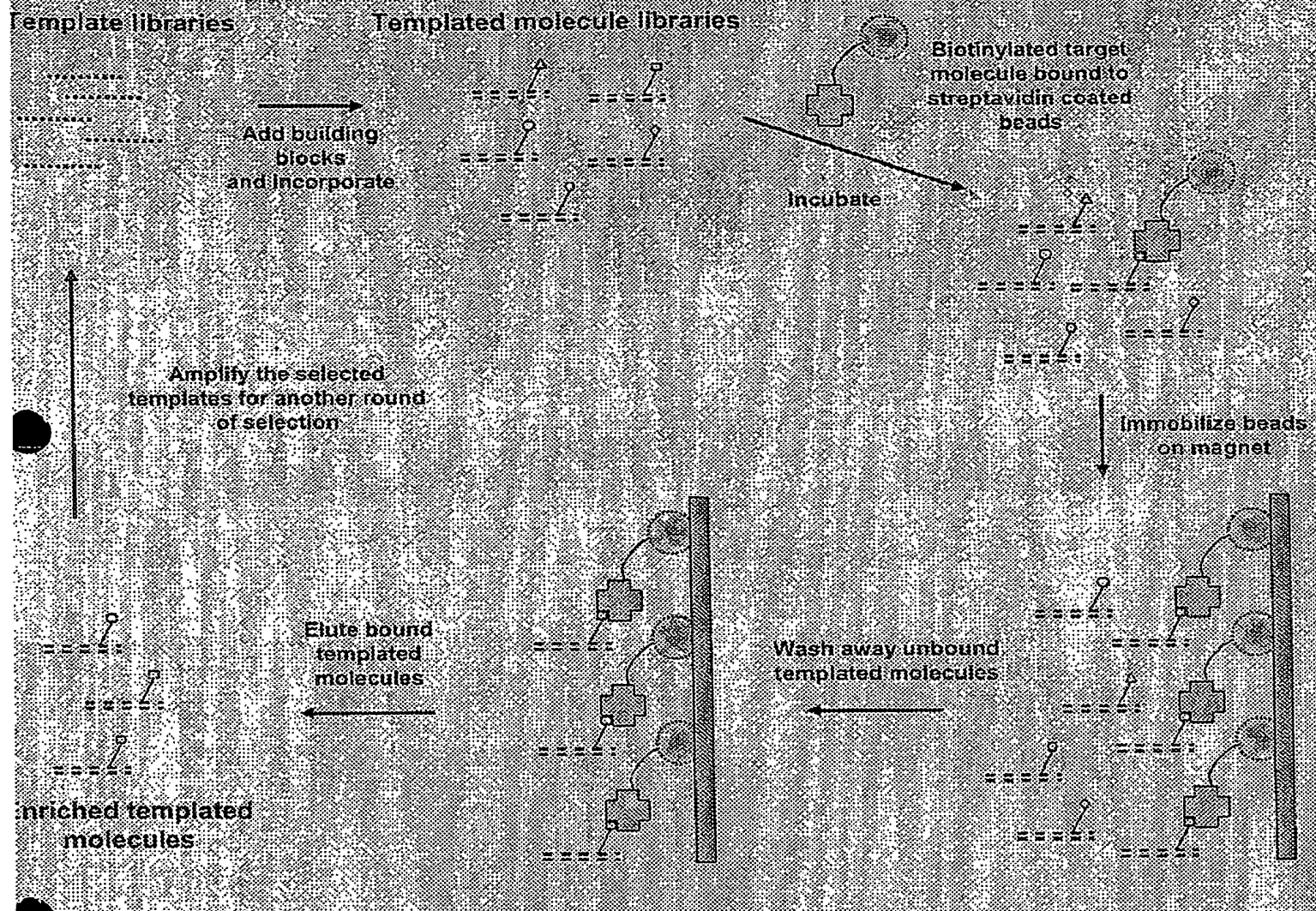


Fig 12.

A typical panning protocol for selection of templated molecules



BEST AVAILABLE COPY